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(54) Title: COMPOUNDS AND METHODS FOR INDUCING GROWTH ARREST AND APOPTOSIS

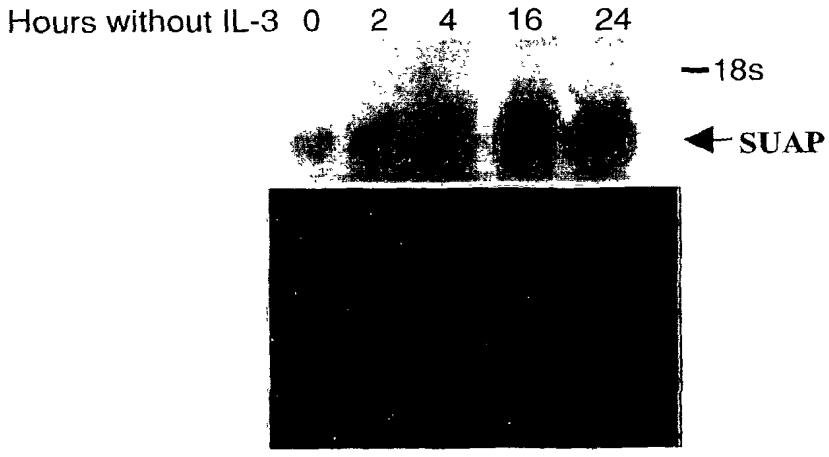


FIG. 6D

(57) Abstract: Growth arrest and apoptosis in cells can be induced in cells which are resistant to apoptosis with SUAP and derivatives, homologs and analogs of SUAP. Detection of endogenous SUAP expression can also be used as a marker of apoptosis in cells undergoing apoptosis-inducing therapeutic treatments.

WO 03/064616 A2

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

COMPOUNDS AND METHODS FOR INDUCING GROWTH ARREST AND APOPTOSIS

Field of the Invention

This invention relates to the induction of growth arrest and apoptosis in cells, in particular cancer cells.

Background of the Invention

Apoptosis, or programmed cell death, is a genetically encoded and tightly regulated process in which the cell actively participates in its own destruction. During apoptosis, the cell undergoes a defined set of morphological changes, including membrane blebbing, condensation of the cytoplasm and chromatin, and DNA fragmentation (Williams GT et al. (1992), *Trends Cell Biol.*, 2: 263-265; Williams GT et al. (1993), *Cell* 74: 777-778; Cotter TG et al. (1994), *Immunol. Rev.* 142: 93-112; Strasser A et al. (2000), *Ann. Rev. Biochem.* 69: 217-245). The cell ultimately collapses into membrane-bounded apoptotic bodies which contain the condensed organelles and chromatin. These apoptotic bodies are eliminated from the body by phagocytic cells. As apoptotic pathways are genetically conserved across histologic and phylogenetic boundaries, the same pathways are common to a variety of cell types in different species.

Cells can undergo apoptosis as part of a growth or developmental plan. For example, the formation of the fingers and toes in the fetus requires the removal, by apoptosis, of cells in the tissue between them. Also, the sloughing off of the inner lining of the uterus at the start of each menstrual cycle occurs by apoptosis of uterine endometrial cells.

Apoptosis is also used to remove cells which pose a threat to an organism. For example, cells with damaged DNA can undergo apoptosis to prevent the propagation of deleterious mutations, or to remove potentially malignant cells. Likewise, abnormally proliferating cells can undergo apoptosis after a certain number of cell divisions.

However, certain proliferating cells escape elimination by apoptosis and can eventually cause a pathological condition in the body. Some clinically

relevant examples of this are the uncontrolled growth of cancer cells to cause a tumor, or the abnormal proliferation of hematopoietic cells in the leukemias. Other examples include the uncontrolled growth of cells involved in non-cancerous proliferative diseases.

In particular, perturbation of apoptosis is evident in cell lines derived from patients suffering from chronic myelogenous leukemia (CML). The hallmark of these cells is the presence of the Philadelphia chromosome, which results from a translocation between chromosomes 9 and 22 that generates the *bcr-abl* chimeric oncoprotein (Nowell PC (1974), *Ann. Clin. Lab. Sci.* 4: 234-240). *Bcr-abl* positive cell lines are extraordinarily resistant to numerous apoptotic stimuli, including withdrawal of the apoptosis-inhibiting cytokine IL-3.

It is therefore desirable to identify compounds which overcome the resistance to apoptosis exhibited by tumor, leukemic, and other abnormally proliferating cells, so that these cells can be eliminated from the body. Overcoming these cells' resistance to apoptosis can be achieved through manipulation of molecular regulators of apoptosis, in particular by the introduction of factors into the cell which promote the apoptotic response.

Promoters of apoptosis sometimes induce a period of growth arrest in the cell, during which the cell "decides" to undergo programmed cell death or some other process, such as differentiation into a certain cell type. Events which occur during this period of growth arrest can increase the likelihood of the cell proceeding down an apoptotic pathway. For example, the introduction of an external apoptotic stimulus, such as radiation or anti-cancer drugs, can encourage growth-arrested cells to enter apoptosis. However, the growth-arrested cell can undergo apoptosis without further external stimuli.

The molecular "decision" to undergo apoptosis is dependent upon the ratio of inducer to suppressor proteins inside the cell (reviewed in Strasser et al (2000), *supra* and Reed JC (1998), *Oncogene* 17: 3225-3236). Promoters of apoptosis can affect the balance of apoptosis inducer and suppressor proteins, for example by transcriptional regulation of the genes which produce these proteins. One known promoter of apoptosis, p53, upregulates certain apoptotic inducers and downregulates certain apoptotic suppressors (White E (1995),

Genes. Dev. 10: 1-15; Hoffman B et al. (1994), *Oncogene* 9: 1807-1812; Liebermann DA et al (1995), *Oncogene* 11: 199-210; Ko et al. (1996), *TIBS* 20: 426-430). p53 can also promote apoptosis in cells with damaged DNA by stalling the cell cycle in either the G1 or G2/M phase, so the cell can repair its genetic material (reviewed in Liebermann DA et al (1995), *supra*; Ko et al. (1996), *supra*; Enoch et al. (1995), *TIBS* 20: 426-430; Bates et al. (1996) *Curr. Opin. Genes. Dev.* 6: 12-19; Amundson SA et al. (1998), *Oncogene* 17: 3287-3299). In the event that the necessary repairs cannot be made, apoptosis occurs in order to prevent additional clonal expansion of a cell with a mutated genome.

However, known apoptosis promoters such as p53 do not consistently induce apoptosis in different cell types. For example, myeloid cells are prone to rapidly apoptose in response to ionizing radiation, whereas identically treated fibroblasts tend to die more slowly during the next round of mitosis or by necrosis (Radford IR et al. (1994), *Internat. J. Radiat. Biol.* 65: 217-227).

There is thus a need for apoptosis regulators, and methods of their use, which reliably promote growth arrest and/or apoptosis in cells which are resistant to elimination by apoptosis. There is further need for apoptosis regulators, and methods of their use, which enhance the activity of external apoptotic stimuli in cells which are resistant to elimination by apoptosis.

Summary of the Invention

It has been found that the Small Ubiquinated Apoptotic Protein (hereinafter "SUAP"), and derivatives, homologs and analogs of SUAP, induce growth arrest or apoptosis when introduced into a cell.

The invention provides isolated nucleic acid sequences which encode biologically active derivatives and homologs of SUAP. Thus in one embodiment, the present invention provides an isolated nucleic acid sequence comprising SEQ ID NO: 3, and homologs thereof. The invention also provides isolated nucleic acid sequences complementary to SEQ ID NO: 3, and homologs thereof.

The invention also provides isolated biologically active SUAP derivatives, homologs and analogs. In one embodiment, the SUAP derivative

comprises SEQ ID NO: 6. In a further embodiment, the SUAP derivative comprises SEQ ID NO: 7.

The invention also provides antibodies that bind to specific epitopes on SUAP derivatives, homologs, and analogs, or antigenic fragments thereof. The antibodies may be monoclonal or polyclonal, or an antibody fragment that is capable of specifically binding antigen. Preferably, the antibodies bind to epitopes of the SUAP derivatives, homologs or analogs that are not present in wild-type SUAP.

The invention also provides a hybridoma that produces a monoclonal antibody which specifically binds to the compounds of the invention.

The invention also provides a method of inducing growth arrest or apoptosis in cells, comprising contacting the cells with an effective amount of a compound comprising SUAP or biologically active derivative, homolog or analog thereof, such that the compound is introduced into the cell and growth arrest or apoptosis is effected. The compound can be introduced into the cell by transfecting the cell with a nucleic acid encoding the compound, or by direct introduction of the compound into the cell.

The invention also provides a method of enhancing the effect of an external apoptotic stimulus on cells, comprising the steps of contacting the cells with an effective amount of a compound comprising SUAP or biologically active derivative, homolog or analog thereof, such that the compound is introduced into the cell, and applying an external apoptotic stimulus to the cells.

The invention also provides a method of identifying external stimuli which induce apoptosis in cells, comprising determining the basal level of SUAP expression in the cells, applying an external stimulus to the cells, and determining the level of endogenous SUAP expression in the cell. An increased level of SUAP expression relative to the basal level indicates that the test compound has induced apoptosis in the cells.

Amino Acid Abbreviations

The nomenclature used to describe the peptide compounds of the present invention follows the conventional practice wherein the amino group is

presented to the left and the carboxy group to the right of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino-and carboxy-terminal groups, although not specifically shown, will be understood to be in the form they would assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by a one-letter or three-letter designation, corresponding to the trivial name of the amino acid, in accordance with the following schedule:

A	Alanine	Ala
C	Cysteine	Cys
D	Aspartic Acid	Asp
E	Glutamic Acid	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamine	Gln
R	Arginine	Arg
S	Serine	Ser
T	Threonine	Thr
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

Definitions

A **"heterologous"** nucleic acid or peptide sequence is a sequence not naturally associated with a host cell into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring sequence. When referring to two sequences, the term means that the two sequences have different origins; for example, a mouse nucleic acid coding sequence and a nucleic acid sequence encoding an HIV TAT protein transduction domain are heterologous sequences with respect to each other.

A **"nucleic acid molecule"** or **"nucleic acid sequence"** is a segment of single- or double-stranded DNA or RNA that can be isolated from any source.

In the context of the present invention, the nucleic acid molecule is preferably a segment of DNA.

"Expression vector" as used herein means a nucleic acid sequence, for example a plasmid, capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. An expression vector also typically comprises sequences required for proper translation of the nucleotide sequence. An expression vector comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression vector may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression vector is heterologous with respect to the host; *i.e.*, the particular nucleic acid sequence of the expression vector does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transfection event. The expression of the nucleic acid sequence in the expression vector may be under the control of a constitutive promoter or an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus (*e.g.*, radiation or IPTG). In the case of a multicellular organism, the promoter can also be specific to a particular tissue, organ or stage of development.

"Operably linked" refers to two or more nucleic acid sequences that are related physically or functionally. For example, a promoter or regulatory DNA sequence is said to be "operably linked" to a DNA sequence that codes for an RNA or a protein if the two sequences are situated such that the regulator DNA sequence will affect the expression level of the coding or structural DNA sequence.

A **"coding sequence"** is a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, hnRNA, sense RNA or antisense RNA. Preferably the RNA is then translated in an organism or cell to produce a protein.

A **"promoter"** is an untranslated DNA sequence upstream of the coding region that contains the binding site for RNA polymerase and initiates transcription of the DNA. The promoter region may also include other elements that act as regulators of gene expression.

"Regulatory elements" refer to sequences involved in controlling the expression of a nucleotide sequence. Regulatory elements comprise a promoter operably linked to the nucleotide sequence of interest and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

As used herein, "**SUAP expression**" includes both expression at the RNA (*e.g.*, hnRNA and mRNA) and protein level. Thus, SUAP expression can be confirmed by detecting either the presence of SUAP RNA, SUAP protein, or both. Methods for detecting SUAP RNA and protein in cells are well-known in the art, and include PCR-based techniques, and Northern and Western blot analysis.

"Transfection" is a process for introducing isolated nucleic acid into a host cell or organism. The nucleic acid molecule can be stably integrated into the genome of the host cell or organism, or the nucleic acid molecule can be present as an extrachromosomal molecule. Transfected cells or organisms are understood to encompass not only the end product of a transfection process, but also progeny thereof containing the transfected nucleic acid.

"Transgenic" or **"recombinant"** refer to a host organism or cell into which a heterologous nucleic acid molecule has been transfected. A "non-transfected" "non-transgenic", or "non-recombinant" host refers to a wild-type organism or cell which does not contain the transfected nucleic acid molecule.

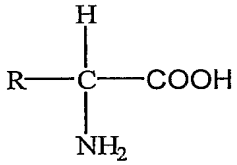
"Antibody" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library.

"Isolated" means altered or removed from the natural state through the actions of a human being. For example, a nucleic acid sequence or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid sequence or peptide partially or completely separated from the coexisting

materials of its natural state is "isolated." An isolated nucleic acid sequence or protein may exist in substantially purified form, or may exist in a non-native environment such as, for example, a host cell.

The expression "**amino acid**" as used herein is meant to include both natural and synthetic amino acids, and both D and L amino acids. **"Standard amino acid"** means any of the twenty standard L-amino acids commonly found in naturally occurring peptides. **"Nonstandard amino acid"** means any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or derived from a natural source. As used herein, "synthetic amino acid" also encompasses chemically modified amino acids, including but not limited to salts, amino acid derivatives (such as amides), and substitutions. Amino acids contained within the peptides of the present invention, and particularly at the carboxy- or amino-terminus, can be modified by methylation, amidation, acetylation or substitution with other chemical groups which can change the peptide's circulating half life without adversely affecting their biological activity. Additionally, a disulfide linkage may be present or absent in the peptides of the invention.

Amino acids have the following general structure:



Amino acids are classified into seven groups on the basis of the side chain R: (1) aliphatic side chains, (2) side chains containing a hydroxylic (OH) group, (3) side chains containing sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an aromatic ring, and (7) proline, an imino acid in which the side chain is fused to the amino group.

"Peptide" and **"protein"** are used interchangeably, and refer to a compound comprised of at least two amino acid residues covalently linked by peptide bonds or modified peptide bonds (*e.g.*, peptide isosteres). No limitation is placed on the maximum number of amino acids which may comprise a

protein or peptide. The amino acids comprising the peptides or proteins described herein and in the appended claims are understood to be either D or L amino acids with L amino acids being preferred. The amino acid comprising the peptides or proteins described herein may also be modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in a peptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It is understood that the same type of modification may be present in the same or varying degrees at several sites in a given peptide. Also, a given peptide may contain many types of modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS--STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al., "Analysis for protein modifications and nonprotein cofactors", Meth Enzymol (1990) 182:626-646 and Rattan et al., "Protein Synthesis: Posttranslational Modifications and Aging", Ann NY Acad Sci (1992) 663:48-62, the disclosures of which are herein incorporated by reference.

As used herein, **"protecting group"** with respect to a terminal amino group of a peptide means any of the various amino-terminal protecting groups traditionally employed in peptide synthesis. Such protecting groups include, for

example, acyl protecting groups such as formyl, acetyl, benzoyl, trifluoroacetyl, succinyl, and methoxysuccinyl; aromatic urethane protecting groups such as benzyloxycarbonyl; and aliphatic urethane protecting groups, for example, tert-butoxycarbonyl or adamantyloxycarbonyl. See Gross and Mienhofer, eds., *The Peptides*, vol. 3, pp. 3-88 (Academic Press, New York, 1981), the entire disclosure of which is herein incorporated by reference, for suitable protecting groups.

As used herein, **"protecting group"** with respect to a terminal carboxy group of a peptide means any of various carboxyl-terminal protecting groups traditionally employed in peptide synthesis. Such protecting groups include, for example, tert-butyl, benzyl or other acceptable groups linked to the terminal carboxyl group through an ester or ether bond.

"Derivative" includes any purposefully generated peptide which in its entirety, or in part, has a substantially similar amino acid sequence to SUAP. Derivatives of SUAP may be characterized by single or multiple amino acid substitutions, deletions, additions, or replacements. These derivatives may include (a) derivatives in which one or more amino acid residues of SUAP are substituted with conservative or non-conservative amino acids; (b) derivatives in which one or more amino acids are added to SUAP; (c) derivatives in which one or more of the amino acids of SUAP include a substituent group; (d) derivatives in which SUAP or a portion thereof is fused to another peptide (*e.g.*, serum albumin, Myc, or a protein transduction domain); (e) derivatives in which one or more nonstandard amino acid residues (*i.e.*, those other than the 20 standard L-amino acids found in naturally occurring proteins) are incorporated or substituted into the SUAP sequence; and (f) derivatives in which one or more nonamino acid linking groups are incorporated into or replace a portion of SUAP.

A **"homolog"** of SUAP includes any nonpurposely generated peptide which in its entirety, or in part, has a substantially similar amino acid sequence to SUAP. Homologs may include paralogs, orthologs, and naturally occurring alleles or variants of SUAP.

An **"analog"** of SUAP includes any non-peptide molecule comprising a structure that mimics the physico-chemical and spatial characteristics of SUAP, and is biologically active.

"Biologically active," with respect to SUAP, or fragments, derivatives, homologs and analogs of SUAP means the ability of the compound to induce growth arrest or apoptosis in a cell, or that the compound exhibits immunogenic characteristics of a SUAP epitope. Growth arrest in a cell or population of cells can be determined by the cell viability and flow cytometry assays described in Example 7 below. Apoptosis in a cell or population of cells can be determined by detecting the characteristic endonucleolytic cleavage of DNA, as described in Example 5 below.

A compound which **"exhibits immunogenic characteristics of a SUAP epitope"** means that the compound 1) elicits a specific humoral or cellular immune response in a mammal to an epitope of SUAP. As used herein, an **"epitope"** is a distinct structural area of an immunogen that can combine with an antibody or T-lymphocyte receptor. Reactivity to SUAP epitopes may be determined by known immunological techniques, such as immunoprecipitations and Western blot analyses as described above and in the Examples. By way of illustration, a compound exhibiting immunogenic characteristics of a SUAP epitope will, on injection into a mouse, cause that mouse to develop antibodies that will react with SUAP as detected, for example, by Western blot or enzyme-linked immunosorbent assay.

By **"libraries"** is meant pools and subpools of compounds, for example fragments, derivatives, homologs, analogs, or pro-analogs of SUAP.

"Variant" as the term is used herein, is a nucleic acid sequence or peptide that differs from a reference nucleic acid sequence or peptide respectively, but retains essential properties. Changes in the sequence of a nucleic acid sequence variant may not alter the amino acid sequence of a peptide encoded by the reference nucleic acid, or may result in amino acid substitutions, additions, deletions, fusions and truncations. Changes in the sequence of peptide variants are typically limited or conservative, so that the sequences of the reference peptide and the variant are closely similar overall and, in many regions, identical. A variant and reference peptide may differ in amino acid

sequence by one or more substitutions, additions, deletions in any combination. A variant of a nucleic acid sequence or peptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of nucleic acid sequences and peptides may be made by mutagenesis techniques or by direct synthesis.

As used herein, a peptide or a portion of a peptide which has a **"substantially similar amino acid sequence"** to a reference peptide means the peptide, or a portion thereof, has an amino acid sequence identity or similarity to the reference peptide of greater than about 70%. Preferably, the sequence identity is greater than about 75%, more preferably greater than about 80%, particularly preferably greater than about 90%, and more particularly preferably greater than about 95%, and most preferably greater than about 98%. Amino acid sequence similarity or identity may be computed by using the BLASTP and TBLASTN programs which employ the BLAST (basic local alignment search tool) 2.0.14 algorithm; BLASTP and TBLASTN settings suitable for use in such computations are indicated in Table 1 below. Amino acid sequence identity is reported under "Identities" by the BLASTP and TBLASTN programs. Amino acid sequence similarity is reported under "Positives" by the BLASTP and TBLASTN programs. Techniques for computing amino acid sequence similarity or identity are well known to those skilled in the art, and the use of the BLAST algorithm is described in Altschul et al. (1990), *J. Mol. Biol.* 215: 403-10 and Altschul et al. (1997), *Nucleic Acids Res.* 25:3389-3402, the disclosures of which are herein incorporated by reference in their entirety. BLASTP and TBLASTN programs utilizing the BLAST 2.0.14 algorithm are available, for example, at the National Center for Biotechnology Information World Wide Web site BLAST server.

Table 1 - Settings to be used for the computation of amino acid sequence similarity or identity with BLASTP and TBLASTN programs utilizing the BLAST 2.0.14 algorithm.

Expect Value	10
Filter	Low complexity filtering using SEG program*
Substitution Matrix	BLOSUM62
Gap existence cost	11
Per residue gap cost	1
Lambda ratio	0.85
Word size	3

*The SEG program is described by Wootton and Federhen (1993), *Comput. Chem.* 17: 149-163.

"Substantially similar nucleic acid sequence" means a nucleic acid sequence corresponding to a reference nucleic acid sequence wherein the corresponding sequence encodes a peptide having substantially the same structure and function as the peptide encoded by the reference nucleic acid sequence; *e.g.*, where only changes in amino acids not affecting the peptide function occur. Preferably, the substantially similar nucleic acid sequence encodes the peptide encoded by the reference nucleic acid sequence. The percentage of identity between the substantially similar nucleic acid sequence and the reference nucleic acid sequence is at least about 70%, Preferably, the sequence identity is greater than about 75%, more preferably greater than about 80%, particularly preferably greater than about 90%, and more particularly preferably greater than about 96%, and most preferably greater than about 98%. Substantial similarity of nucleic acid sequences may be determined by comparing the sequence identity of two sequences, for example by physical/chemical methods (*i.e.*, hybridization) or by sequence alignment via computer algorithm. Suitable nucleic acid hybridization conditions to determine if a nucleotide sequence is substantially similar to a reference nucleotide sequence are: 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50 °C with washing in 2X standard saline citrate (SSC), 0.1% SDS at 50 °C; preferably in 7% (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50 °C with washing in 1XSSC, 0.1% SDS at 50 °C, more preferably 7% SDS, 0.5 M $NaPO_4$, 1 mM

EDTA at 50 °C with washing in 0.5.XSSC, 0.1% SDS at 50 °C; and most preferably in 7% SDS, 0.5 M $NaPO_4$, 1 mM EDTA at 50 °C with washing in 0.1XSSC, 0.1% SDS at 65 °C. Suitable computer algorithms to determine substantial similarity between two nucleic acid sequences include the GCS program package (Devereux et al. (1984), *Nucl. Acids Res.* 12: 387), and the BLASTN or FASTA programs (Altschul et al. (1990), *J. Mol. Biol.* 215: 403). The default settings provided with these programs are adequate for determining substantial similarity of nucleic acid sequences for purposes of the present invention.

"Substantially purified" refers to a peptide or nucleic acid molecule which is substantially homogenous in character due to the removal of other compounds (*e.g.*, other peptides, nucleic acids, carbohydrates, lipids) or other cells originally present. "Substantially purified" is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of impurities which do not interfere with biological activity, and which may be present, for example, due to incomplete purification, addition of stabilizers, or formulation into a pharmaceutically acceptable preparation.

"Synthetic mutant" includes any purposefully generated mutant or variant derived from SUAP. Such mutants may be purposefully generated by, for example, chemical mutagenesis, polymerase chain reaction (PCR) based approaches, or primer-based mutagenesis strategies well known to those skilled in the art.

Brief Description of the Figures

Figure 1 is the primary amino acid sequence of SUAP. All lysine (K) residues that may serve as potential ubiquitination sites are underlined.

Figure 2 is an autoradiogram of a commercial multi-tissue Northern blot (Clontech) probed with the full length SUAP cDNA. The size of the molecular weight marker (in kb) is indicated on the right.

Figure 3A is an autoradiogram of a Western blot showing detection of HA epitope-tagged SUAP extracted from control cells (vector) and COS-7 cells transfected with SUAP-expressing plasmid (pSG5-SUAP/HA) and treated with

MG132 at the concentrations and times indicated. **Figure 3B** is a prolonged exposure of the autoradiogram of Figure 3A. **Figure 3C** is an autoradiogram of a Western blot showing detection of HA epitope-tagged SUAP extracted from control cells (vector) and COS-7 cells transfected with SUAP-expressing plasmid (pSG5-SUAP/HA) and treated with Lactacystin or *clasto*-Lactacystin-ß-lactone at the concentrations and time indicated.

Figure 4A is a plot of total viable 32Dcl3 cells vs. days in growth medium containing G-CSF, as determined by trypan blue exclusion. **Figure 4B** shows Cytospin preparations of 32Dcl3 cells grown in the presence of G-CSF for the days indicated, and stained with Wright's Modified and Giemsa stains to show cytoplasmic and nuclear morphology. B=myeloblasts; M=myelocytes; MM=metamyelocytes; G=granylocytes.

Figure 5 is an autoradiogram of a Northern blot showing expression of SUAP RNA during G-CSF-induced terminal granulocytic differentiation of 32Dcl3 cells. 18s and 28s ribosomal RNA are shown as a control for RNA loading.

Figure 6A is a plot showing proliferation of 32Dcl3 cells in response to IL-3. **Figure 6B** is a plot showing the effect of IL-3 deprivation on the growth and apoptosis of 32Dcl3 cells. **Figure 6C** is a photograph of an agarose gel showing low molecular weight cellular DNA extracted from IL-3-deprived 32Dcl3 cells. **Figure 6D** is an autoradiogram of a Northern blot showing induction of SUAP RNA during IL-3 withdrawal-induced apoptosis of 32Dcl3 cells. 18s and 28s ribosomal RNA are shown as a control for RNA loading.

Figure 7A is a plot of cell viability vs. days exposed to DMSO for 32D/*bcr-abl* and 32D/*v-abl* cells. **Figure 7B** is an autoradiogram of a Northern blot showing SUAP RNA induction in 32D/*v-abl* and 32D/*bcr-abl* cells in response to treatment with 1.5% DMSO. 18s and 28s ribosomal RNA are shown as a control for RNA loading.

Figure 8A is an autoradiogram of a Northern blot showing inducible expression of SUAP RNA in 32Dcl3 clones transfected with an IPTG-inducible SUAP expression vector. 18s and 28s rRNA are shown as a loading control. **Figure 8B** shows plots of cell number vs. days exposed to IL-3 for each of three 32Dcl3 clones transfected with an IPTG-inducible SUAP expression vector.

Figure 9 shows autoradiograms of Northern blots of SUAP RNA induced in MCF7 cells in response to **(A)** serum-withdrawal-induced apoptosis; **(B)** taxol-induced apoptosis; **(C)** etoposide-induced apoptosis; **(D)** cisplatin-induced apoptosis; **(E)** camptothecin-induced apoptosis. 18s and 28s ribosomal RNA are shown as a control for RNA loading in each figure.

Figure 10 shows autoradiograms of a Northern blots showing SUAP RNA induced in response to **(A)** irradiation of DU145 and LnCap prostate tumor cells; **(B)** androgen ablation of LnCap cells; and **(C)** irradiation of androgen depleted LnCap cells. 18s and 28s ribosomal RNA are shown as a control for RNA loading in each figure.

Figure 11A is an autoradiogram showing levels of wild-type SUAP and SUAP derivatives over time in COS-7 cells transiently transfected with HA-tagged SUAP expression vectors. **Figure 11B** is a plot showing the stability of wild-type SUAP and SUAP derivatives in transfected COS-7 cells.

Detailed Description of the Invention

SUAP is a peptide of approximately 13 kDa which is expressed in multiple tissues, including heart, brain, testis, liver, kidney, lung spleen, and neoplastic tissues. Endogenous SUAP is up-regulated during apoptosis at both the RNA and protein level, and SUAP protein is rapidly degraded by the proteosome within the cell. Contacting cells with exogenous SUAP, or derivatives, homologs, and analogs thereof, induces growth arrest and apoptosis in cells and enhances the effect of external apoptosis-inducing stimuli.

A mouse SUAP cDNA sequence has been reported previously (see GenBank record accession no. BC00971, the disclosure of which is herein incorporated by reference), but heretofore no function has been elucidated for the encoded protein. A SUAP cDNA clone of approximately 1.1 kb has been isolated from mouse 32Dcl3 cells undergoing apoptosis as a result of IL-3 deprivation (see Example 1), and is disclosed in SEQ ID NO: 1. The cDNA of SEQ ID NO: 1 differs from the cDNA reported in GenBank record accession no. BC00971 in the 3' and 5' untranslated regions, but both cDNAs appear to have identical coding regions.

The SUAP cDNA of SEQ ID NO: 1 shows a single large open reading frame of approximately 360 nucleotides encoding a protein of 119 amino acids. The deduced amino acid sequence of SUAP is given in FIG. 1 and in SEQ ID NO: 2.

A human SUAP cDNA sequence has also been reported, see GenBank record accession no. BC005009, the disclosure of which is herein incorporated by reference. Again, no function has heretofore been reported for the encoded protein. This cDNA sequence of human SUAP is given in SEQ ID NO: 3, and the deduced amino acid sequence is given in SEQ ID NO: 4.

The present invention also provides novel peptide derivatives of SEQ ID NO: 2 and SEQ ID NO: 4, in particular synthetic mutants of SEQ ID NO: 2 and SEQ ID NO: 4, which exhibit SUAP biological activity. These SUAP synthetic mutants can be designed so that the stability of the protein inside the cell is altered; *i.e.*, increased or decreased with respect to wild-type SUAP.

Exemplary SUAP synthetic mutants include SUAP fusion proteins. For example, one or more extra amino acid sequences can be introduced into the wild-type SUAP sequence at any point or points which allow the fusion protein to retain SUAP biological activity. In a preferred embodiment, the extra amino acid sequences are attached directly to the N- and/or C-termini of SUAP, or are inserted within 1 to 3 amino acids of the N- or C-terminal amino acid. For example, an extra amino acid sequence can be introduced into the N-terminal portion of the SUAP protein immediately after the initial methionine. The extra amino acid sequences can comprise a peptide leader sequence that directs entry of the protein into a cell. Such leader sequences include "protein transduction domains" or "PTDs", as discussed in more detail below. The extra amino acid sequences can also comprise sequences which confer greater stability to the fusion protein as compared to wild-type SUAP.

The extra amino acid sequences introduced into the SUAP sequence can be any length which allows the fusion protein to retain SUAP biological activity. Preferably, the length of each extra amino acid sequence is from 1 to 50 amino acids in length, more preferably from 1 to 20 amino acids in length, most preferably from 1 to 12 amino acids in length. Particularly preferred are extra amino acid sequences of 11, 12, or 13 amino acids in length.

SUAP fusion proteins can be generated using standard techniques known in the art, for example by subcloning nucleic acid sequences encoding SUAP and a heterologous peptide sequence into the same expression vector, such that the SUAP and heterologous sequences are expressed together in the same protein. In particular, oligonucleotide-directed fusion PCR can be performed on wild-type SUAP to insert nucleic acid sequences encoding one or more extra amino acid sequences at the desired position(s) in wild-type SUAP (see Example 18 below). Other techniques for obtaining SUAP fusion proteins are known to persons having ordinary skill in the art and include, for example, solid phase peptide synthesis techniques and chemical synthetic techniques as described below.

Linking groups may also be used to join or replace portions of SUAP and other peptides. Linking groups include, for example, cyclic compounds capable of connecting an amino-terminal portion of an extra amino acid sequence and a carboxyl terminal portion of SUAP.

A preferred SUAP fusion protein comprises a wild-type SUAP sequence with a 12-amino acid Myc epitope inserted immediately after the initial methionine (hereinafter the "Myc-SUAP fusion protein"). The cDNA sequence encoding the Myc-SUAP fusion protein, which also has an hemagglutinin (HA) tag at the C-terminal end, is given in SEQ ID NO: 5, and the encoded amino acid sequence is given in SEQ ID NO: 6. As shown in Example 18 and Fig. 11B below, the Myc-SUAP/HA fusion protein is more stable within the cell than wild-type SUAP.

Other SUAP synthetic mutants include "lysine to arginine" (K→R) mutants, in which one or more of the lysines in the wild-type SUAP sequence are converted to arginine. These lysines are believed to act as ubiquitination sites, and conversion of lysine to arginine abolishes a cell's ability to ubiquinate the protein at a given site.

K→R mutants can be generated using standard techniques known in the art. For example, oligonucleotide-directed mutagenesis can be performed on wild-type SUAP to systematically and progressively substitute arginine for each lysine residue (see Example 18 below). In a preferred K→R mutant, the lysines at positions 6, 8, 39, 83, 89, 99, 101, 104 and 114 in wild-type SUAP are

converted to arginines (see SEQ ID NO: 7). Abolishing these potential ubiquitination sites increases this protein's stability inside the cell in comparison to wild-type SUAP (see Example 18 and FIG. 11B).

Other techniques for generating SUAP derivatives are as described in U.S. patent 6,030,942, the entire disclosure of which is herein incorporated by reference (derivatives are designated "peptoids" in the 6,030,942 patent). SUAP derivatives may also incorporate labels such as are described below into their structure.

The present invention also provides biologically active homologs of SUAP. SUAP homologs have both a substantially similar amino acid sequence and can induce growth arrest or apoptosis, and can be identified on this basis. SUAP homologs can also incorporate labels such as are described below into their structure. For example, the biological activity of SUAP homologs can be determined by the growth arrest and apoptosis assays set forth in Examples 5 and 7 below.

The present invention also provides biologically active analogs of SUAP. SUAP analogs can, for example, be small organic molecules capable of inducing growth arrest or apoptosis in cells. SUAP analogs can also incorporate labels such as are described below into their structure.

SUAP analogs preferably comprise a structure, called a pharmacophore, that mimics the physico-chemical and spatial characteristics of SUAP. Consequently, pro-analogs of SUAP can be designed based on variations in the molecular structure of SUAP or portions of SUAP. The biological activity of SUAP analogs can be determined, for example, through the growth arrest and apoptosis assays set forth in Examples 5 and 7 below. Alternatively, SUAP structure and function can be determined using nuclear magnetic resonance (NMR), crystallographic, or computational methods which permit the electron density, electrostatic charges or molecular structure of certain portions of SUAP to be mapped.

The solid-phase synthesis methods described by Schreiber (2000), Science 287: 1964-1969, the entire disclosure of which is herein incorporated by reference, can be used to generate a library of distinct pro-analogs generated by organic syntheses. Briefly, a suitable synthesis support, for example a resin, is

coupled to a pro-analog precursor and the pro-analog precursor is subsequently modified by organic reactions such as, for example, Diels-Alder cyclization. The immobilized pro-analog is then be released from the solid substrate. Pools and subpools of pro-analogs can be generated by automated synthesis techniques in parallel, such that all synthesis and resynthesis can be performed in a matter of days; pools and subpools of pro-analogs are said to comprise libraries. Once generated, pro-analog libraries can be screened for analogs; *i.e.* compounds exhibiting the ability to induce growth arrest or apoptosis in cells, for example as described below Examples 5 and 7.

Pro-analogs of SUAP can also be designed, for example, by using the retrosynthetic, target oriented, or diversity-oriented synthesis strategies described by Schreiber (2000), *supra*. Retrosynthetic strategies require that key structural elements in a molecule be identified and then incorporated into the structure of otherwise distinct pro-analogs generated by organic syntheses. U.S. patent 6,030,942, in particular Example 4 therein, describes retrosynthetic methods for the design and selection of analogs based on identified key structural elements in a protein, and is incorporated herein in its entirety.

SUAP, or biologically active derivatives and homologs of SUAP, comprise natural or synthetic peptides produced by any known means, including synthesis by biological systems and by chemical methods.

Biological synthesis of peptides is well known in the art, and includes the transcription and translation of a synthetic gene encoding SUAP or biologically active derivatives or homologs thereof. Chemical peptide synthesis includes manual and automated techniques well known to those skilled in the art.

For example, the nucleic acid sequences of SEQ ID NOS: 1, 3 or 5 can be subcloned into an appropriate plasmid expression vector for propagation and expression in an appropriate host. The techniques used to isolate or construct nucleic acid sequences, construct plasmid expression vectors, transfect host cells, and express a nucleic acid sequence of interest are widely practiced in the art, and practitioners or ordinary skill are familiar with the standard resource materials which describe specific conditions and procedures. General methods for the cloning and expression of recombinant molecules are described in, for

example, Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratories, 1982; and in Ausubel, Current Protocols in Molecular Biology, Wiley and Sons, 1987, the disclosures of which are incorporated herein by reference.

Proteins produced from an expression vector may be obtained from the host cell by cell lysis, or by using heterologous signal sequences fused to the protein which cause secretion of the protein into the surrounding medium. Preferably, the signal sequence is designed so that it may be removed by chemical or enzymatic cleavage, as is known in the art. SUAP or derivatives and homologs of SUAP thus produced may then be purified in a manner similar to that utilized for isolation of SUAP from natural sources.

SUAP, and derivatives and homologs thereof, can also be synthesized *de novo* using conventional solid phase synthesis methods. In such methods, the peptide chain is prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various N-protecting groups, *e.g.*, the carbobenzyloxy group or the *t*-butyloxycarbonyl group; various coupling reagents *e.g.*, dicyclohexylcarbodiimide or carbonyldimidazole; various active esters, *e.g.*, esters of N-hydroxyphthalimide or N-hydroxy-succinimide; and the various cleavage reagents, *e.g.*, trifluoroactetic acid (TFA), HCl in dioxane, boron tris-(trifluoracetate) and cyanogen bromide; and reaction in solution with isolation and purification of intermediates are methods well-known to those of ordinary skill in the art.

A preferred peptide synthesis method follows conventional Merrifield solid phase procedures well known to those skilled in the art. Additional information about solid phase synthesis procedures can be had by reference to Steward and Young, Solid Phase Peptide Synthesis, W.H. Freeman & Co., San Francisco, 1969; the review chapter by Merrifield in Advances in Enzymology 32:221-296, (Nold FF, ed.), Interscience Publishers, New York, 1969; and Erickson and Merrifield (1990), *The Proteins* 2: 61-64, the entire disclosures of which are incorporated herein by reference. Crude peptide preparations resulting from solid phase syntheses may be purified by methods well known in the art, such as preparative HPLC. The amino-terminus may be protected

according to the methods described for example by Yang *et al.*, *FEBS Lett.* 272: 61-64 (1990), the entire disclosure of which is herein incorporated by reference.

Automated peptide synthesis with commercially available peptide synthesizers may also be used to produce proteins of the invention, for example SEQ ID NOS: 2, 4, 6 and 7.

SUAP, and derivatives and homologs thereof, can also comprise a label; *i.e.*, substances which are detectable. Suitable labels include compounds which are magnetic resonance active (*e.g.*, chelates of gadolinium and iron nanoparticles); radiodense (*e.g.*, iodinated phenyl compounds); fluorescent (*e.g.*, fluorescein isothiocyanate [FITC] and fluorescent proteins originating from *Renilla reniformi*); radioactive (*e.g.*, radionuclides such as ^{32}P, ^{33}P, ^{35}S, ^{125}I, or ^{123}I); detectable by ultrasound; and detectable by visible, infrared or ultraviolet light (*e.g.*, peptide chromophores such as phycoerythrin or phycocyanin and the like, and bioluminescent peptides such as the luciferases originating from *Photinus pyrali*). For example, the label may comprise an NH_2-terminal FITC-Gly-Gly-Gly-Gly motif that is conjugated to a protein transduction domain.

Methods of modifying peptide sequences with labels are well known to those skilled in the art. For example, methods of conjugating fluorescent compounds such as fluorescein isothiocyanate to short peptides are described in Danen *et al.*, *Exp. Cell Res.*, 238:188-86 (1998), the entire disclosure of which is herein incorporated by reference.

The invention also provides nucleic acid sequences encoding SUAP derivatives or homologs. In one embodiment, such a nucleic acid sequence comprises SEQ ID NO: 5. The invention also encompasses complementary sequences of SEQ ID NO: 5, and homologous nucleic acid sequences substantially similar to the complete sequence of SEQ ID NO: 5 and/or its complement. Such sequences include DNA, RNA, and analogs thereof, including peptide nucleic acids.

The invention also provides nucleic acid sequences substantially similar SEQ ID NOS: 1, 3 or 5, which encode peptides that are the same or similar to SUAP, or to derivatives and homologs of SUAP. One of ordinary skill in the art may readily identify nucleic acid sequences which encode SUAP, or derivatives or homologs thereof, based on substantial similarity to SEQ ID NOS: 1, 3 or 5.

Nucleic acid sequences that exhibit substantial similarity to SEQ ID NOS: 1, 3 or 5 may be considered SUAP, SUAP derivative or SUAP homolog nucleic acid sequences according to the present invention.

The nucleotide sequences described herein may be used to produce recombinant SUAP, SUAP derivative or SUAP homolog amino acid sequences which can be used in the methods of the invention.

The present invention also provides antibodies against SUAP derivatives, homologs, analogs, or antigenic fragments thereof. The antibody of the invention can, for example, specifically bind an epitope of a SUAP derivative, homolog, or analog. The antibody may be a monoclonal antibody or a polyclonal antibody or an antibody fragment that is capable of binding antigen. Preferably, the antibodies specifically bind an epitope of SEQ ID NOS: 6 and 7 which is not present in wild-type SUAP. Such antibodies bind to SEQ ID NOS: 6 and 7 or an immunogenic fragment thereof, but not to SEQ ID NOS: 2 or 4, or an immunogenic fragment thereof.

The antibodies of the invention can, for example, comprise polyclonal antibodies, and preparations thereof, produced by immunizing an animal with substantially pure SUAP derivative, homolog or analog, or an immunogenic fragment thereof according to standard techniques. The present invention includes chimeric, single chain, and humanized antibodies, as well as Fab fragments and the products of a Fab expression library. Antibody fragments, such as Fab antibody fragments, which retain some ability to selectively bind to the antigen of the antibody from which they are derived, can be made using well-known methods in the art. Such methods are generally described in U.S. patent 5,876,997, the entire disclosure of which is incorporated herein by reference.

The antibodies of the invention can also comprise monoclonal antibodies, for example as prepared by the method of Mishell, B.B. et al., Selected Methods In Cellular Immunology, (Freeman WH, ed.) San Francisco, 1980, the entire disclosure of which is herein incorporated by reference. Briefly, a SUAP derivative, homolog or analog, or an immunogenic fragment thereof is used to immunize spleen cells of Balb/C mice, which are then fused with myeloma cells. Fused cells containing spleen and myeloma cell

characteristics are isolated by growth in HAT medium, a medium which kills both parental cells but allows the fused products to survive and grow. Hybridomas producing monoclonal antibodies that specifically bind to a SUAP derivative, homolog or analog, or an immunogenic fragment thereof are isolated.

Antibodies of the invention can be used to purify SUAP derivatives and homologs, using immunoaffinity techniques which are well known by those of skill in the art.

Compounds comprising SUAP, and derivatives, homologs and analogs of SUAP are useful in inducing growth arrest or apoptosis in cells. In the practice of the present invention, growth arrest or apoptosis can be induced by contacting the cells with SUAP, or a derivative, homolog or analogs of SUAP so that the compound is introduced into the cell.

As used herein, "contacting" a cell with a compound of the invention includes any method for introducing the present compounds into the cytoplasm of a cell. Such methods include transfection of nucleic acid sequences encoding SUAP, or a biologically active derivative or homolog thereof into a cell such that the compounds are expressed therein. Such methods also include the direct introduction of exogenous proteins or other compounds into a cell. Both cultured cells and cells in a living organism can be contacted with the present compounds.

As discussed above, SUAP is a ubiquitously expressed apoptotic regulator protein which is part of a cellular pathway found in virtually all eukaryotic cells. Thus, the present compounds can induce growth arrest or apoptosis in virtually any eukaryotic cell type, including cell types which are resistant to normal internal or external apoptotic stimuli. In particular, the present compounds are useful in inducing growth arrest and apoptosis in a broad spectrum of cancer cell types of diverse histologic subtype and origin.

Cancer cell types which undergo growth arrest or apoptosis when contacted with the present compounds include those from the cancers listed and described in the National Cancer Institute's "CancerNet" Internet website.

For example, the compounds of the invention can induce growth arrest or apoptosis in primary or metastatic tumor or neoplastic cells in cancers of at

least the following histologic subtypes: sarcoma (cancers of the connective and other tissue of mesodermal origin); melanoma (cancers deriving from pigmented melanocytes); carcinoma (cancers of epithelial origin); adenocarcinoma (cancers of glandular epithelial origin); cancers of neural origin (glioma/glioblastoma and astrocytoma); and hematological neoplasias, such as leukemias and lymphomas (*e.g.*, acute lymphoblastic leukemia, chronic lymphocytic leukemia, and chronic myelocytic leukemia).

The present compounds can also induce growth arrest or apoptosis in primary or metastatic tumor or neoplastic cells from cancers having their origin in at least the following organs or tissues, regardless of histologic subtype: breast; tissues of the male and female urogenital system (*e.g.* ureter, bladder, prostate, testis, ovary, cervix, uterus, vagina); lung; tissues of the gastrointestinal system (*e.g.*, stomach, large and small intestine, colon, rectum); exocrine glands such as the pancreas and adrenals; tissues of the mouth and esophagus; brain and spinal cord; kidney (renal); pancreas; hepatobiliary system (*e.g.*, liver, gall bladder); lymphatic system; smooth and striated muscle; bone and bone marrow; skin; and tissues of the eye.

Furthermore, the present compounds are useful in inducing growth arrest or apoptosis in cells from cancers or tumors in any prognostic stage of development, as measured, for example, by the "Overall Stage Groupings" (also called "Roman Numeral") or the Tumor, Nodes, and Metastases (TNM) staging systems. Appropriate prognostic staging systems and stage descriptions for a given cancer are known in the art, for example as described in the National Cancer Institute's "CancerNet" Internet website.

The present compounds are also useful in the inducing growth arrest or apoptosis in cells from non-cancerous proliferative disorders. Non-cancerous proliferative disorders are characterized by the uncontrolled growth of cells with a benign phenotype, meaning that the cells evade the normal controls on growth but cannot metastasize. Non-cancer proliferative disorders include: hemangiomatosis in the newborn; secondary progressive multiple sclerosis; chronic progressive myelodegenerative disease; neurofibromatosis; ganglioneuromatosis; keloid formation; Paget's Disease of the bone; fibrocystic

disease (*e.g.*, of the breast or uterus); sarcoidosis; Peronies and Duputren's fibrosis, cirrhosis, atherosclerosis and vascular restenosis.

Contacting cells with a compound of the invention can comprise transfecting the cells with a nucleic acid encoding SUAP, or a biologically active derivative or homolog thereof. In one embodiment, the nucleic acid sequence comprises a plasmid expression vector. Such plasmids can be generated by recombinant nucleic acid and molecular cloning techniques well known in the art, as discussed above.

Methods for transfecting eukaryotic cells with nucleic acid sequences are as also well known in the art, and include direct injection of the nucleic acid into the nucleus or pronucleus; electroporation; liposome transfer; receptor mediated nucleic acid delivery, bioballistic or particle acceleration; and transfection mediated by viral vectors. In a preferred method, the transfection is performed with a liposomal transfer compound, e.g., DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, Boehringer - Mannheim) or an equivalent, such as LIPOFECTIN. The amount of nucleic acid used is not critical to the practice of the invention; acceptable results may be achieved with 10 mM nucleic acid/10^5 cells. A ratio of about 500 nanograms of plasmid vector in 3 micrograms of DOTAP per 10^5 cells may be used.

An "effective amount" of a compound comprising SUAP, or a biologically active derivative or homolog thereof which is introduced into a cell via a transfected nucleic acid means any amount of the compound which produces detectable expression within the cell. Expression of the present compounds may be detected by any known technique, such as PCR amplification of mRNA, Northern or Western blot analysis, or by detecting a state of growth arrest or apoptosis in one or more of the transfected cells.

Contacting cells with a compound of the present invention can also comprise the direct introduction of the compounds into cells by methods known in the art. For cultured cells, such methods include administering the compounds either directly to the cells, or in the culture media. Preferably, an effective amount of a compound of the invention is included in fresh growth media which is periodically given to cells growing in culture. For example, a concentrated solution of the present compounds in a carrier such as sterile water,

saline, growth media or the like may be diluted into fresh growth media prior to applying the fresh growth media to the cultured cells.

Cells in a living organism can be contacted with an effective amount of the present compounds by any parenteral or enteral means, such as direct injection into the tissue, injection into the vasculature, oral administration, and the like. A preferred method is the direct injection of the compound into the tissue.

An "effective amount" of a compound comprising SUAP, or a biologically active derivative, homolog or analog thereof which is directly introduced into a cell means any amount of the compound sufficient to cause a state of growth arrest or apoptosis in one or more of the cells. For example, in cultured cells, an effective amount can be between about 25 nM and about 300 nM, preferably at least 50 nM, more preferably at least 100 nM, and particularly preferably at least 150 nM. For *in vivo* administration, an effective amount can be, for example, between about 5g compound/kg and 25g compound/kg, preferably at least 10g compound/kg, more preferably at least 20g compound/kg.

In one embodiment, the present compounds are modified to enhance the uptake of the compound into the cell. For example, the compounds can be encapsulated in a liposome prior to being contacted with the cells. The liposome-encapsulated compounds are delivered directly into the cells by fusion of the liposome to the cellular membrane. Reagents and techniques for encapsulating the present compounds in liposomes are well-known in the art, and include, for example, the ProVectin™ Protein Delivery Reagent from Imgenex.

In another embodiment, the present compounds are modified to enhance their entry into a cell by associating the compounds with a peptide leader sequence known as a "protein transduction domain" or "PTD." These sequences direct entry of the compound into the cell by a process known as "protein transduction." *See* Schwarze et al. (1999), *Science* 285: 1569 – 1572. Proteins ranging in size from 15 to 120 kD have been transduced into a wide variety of human and murine cell types *in* vivo and *in vitro* using the PTD method. *See* Schwarze et al., *supra*; Nagahara et al. (1998), *Nature Med.* 4:

1449; Ezhevsky et al. (1997), *Proc. Natl. Acad. Sci. U.S.A.* 94: 10699; Lissy et al. (1998), *Immunity* 8: 57; and Gius et al. (1999), *Cancer Res.* 59: 2577, the entire disclosures of which are herein incorporated by reference.

Without wishing to be bound by a particular theory, entry of exogenously added, PTD-associated compounds into the cell during protein transduction appears to occur in a rapid, concentration-dependent fashion. Moreover, the process appears to be receptor and transporter independent, *see* Derossi et al. (1996), *J. Biol. Chem.* 271: 18188, and may directly involve the lipid bilayer component of the cell membrane. Thus, all cell types, in particular mammalian cell types, are susceptible to protein transduction. Exogenous administration of PTD-linked compounds to cells results in the rapid delivery of a roughly equal amount of the compound to each cell.

PTDs are well-known in the art, and can comprise any of the known PTD sequences including, for example, arginine-rich sequences such as a peptide of nine to eleven arginine residues, optionally in combination with one to two lysines or glutamines, as described in Guis et al. (1999), *Cancer Res. 59: 2577-2580*, the entire disclosure of which is incorporated herein by reference. Preferred are sequences of eleven arginine residues or the NH_2-terminal 11-amino acid protein transduction domain from the human immunodeficiency virus TAT protein (SEQ ID NO: 8). Other suitable leader sequences include, but are not limited to, other arginine-rich sequences; *e.g.*, 9 to 10 arginines, or six or more arginines in combination with one or more lysines or glutamines. Such leader sequences are known in the art; see, *e.g.*, Guis et al. (1999), *supra.* Preferably, the PTD is designed so that it is cleaved from the compound within the cell. A PTD may be located anywhere on a compound of the invention so long as it does not disrupt the compound's biological activity. For compounds of the invention comprising a peptide, the PTD is preferably located at the N-terminal end.

Reagents and methods for constructing fusion proteins comprising a protein of interest (*e.g.*, SUAP) and a PTD are known in the art; *e.g.*, the Voyager system (Invitrogen Life Technologies), which uses the 38 kDa VP22 protein from Herpes Simplex Virus-1.

It is known that certain external stimuli promote apoptosis in cells. Such stimuli are often employed in the treatment of cancer or non-cancerous proliferative diseases. Often, however, a given external stimulus does not cause a sufficient number of unwanted cells to undergo apoptosis, necessitating multiple and/or more intense treatments. Certain cells may also be resistant to apoptosis. As therapies employing external apoptotic stimuli can also kill normal cells, it is desirable to lessen both the number and intensity of the treatments.

The present compounds are also useful in enhancing the effect of external apoptotic stimuli on cells, particularly cells which are resistant to elimination by apoptosis, so that the number and/or intensity of therapeutic treatments employing the external stimuli can be reduced. Such enhancement is achieved by contacting cells with an effective amount of present compounds prior to, simultaneously with, or after applying an external apoptotic stimulus. Preferably, the present compounds are used to enhance the activity of external apoptotic stimuli in cancer cells and cells from non-cancerous proliferative diseases, as described above.

Cells are preferably contacted with the present compounds prior to application of the external apoptotic stimulus. The present compounds can be administered as much as about 24 hours, preferably no more than about 18 hours, prior to administration of the stimulus. In one embodiment, the present compounds are administered at least about 6 to 12 hours before administration of the external apoptotic stimulus. If the present compounds are administered after the external apoptotic stimulus has been applied, the compounds are preferably administered between within 1 to 6 hours following application of the stimulus.

The specific dose of compound according to the invention needed to obtain enhancement of the external apoptotic stimulus can be determined by the particular circumstances of the individual subject, including the subject's size, weight, age and sex, the nature and stage of the disease being treated, the aggressiveness of the disease, and the type, frequency and intensity of the external apoptotic stimulus. One of ordinary skill in the art is capable of evaluating these factors and choosing an appropriate dose of the present

compounds. For example, a dosage of from about 5 to about 25 g/kg may be utilized. The total dose may be given over multiple administrations. Higher or lower doses are also contemplated.

Examples of external apoptotic stimuli whose activity can be enhanced by the present compounds include any externally applied stress which causes extensive DNA damage and/or prevent DNA repair, or otherwise causes a cell to undergo apoptosis. Such stimuli are known in the art, and include ionizing radiation (*e.g.*, UV, gamma, and high energy beta radiation); chemotherapeutics (*e.g.*, taxol, vincristine, vinblastine, hydroxyurea (HU), cytosine arabinoside (Ara-C), methotrexate, etoposide, camptothecin, cisplatin, doxorubicin, 5-fluorouracil (5FU) and estramustine); administration of agents which directly affect regulators of apoptosis, such as STI571 (a specific tyrosine kinase inhibitor of the BCR-ABL oncoprotein); and deprivation of agents which prevent apoptosis (*e.g.*, hormones or cytokines such as IL-3).

The expression of endogenous SUAP in a cell can indicate that the cell is undergoing growth arrest and/or apoptosis. Thus, the ability of an externally applied stimulus to induce apoptosis in a given cell type can be evaluated by applying a stimulus to the cells, and subsequently assaying the cells for a change in SUAP expression. An increased level of SUAP expression in a cell relative to the basal level of SUAP expression indicates that the stimulus has induced apoptosis. In particular, this method can be used to identify chemical compounds or radiation treatments which are useful in treating cancer and non-cancerous proliferative diseases.

As used herein, "basal level of SUAP expression" in a cell means the level of SUAP expression in a cell which is not undergoing growth arrest or apoptosis. The basal level of SUAP expression can be determined by assaying a portion of a particular population of cells prior to application of the external stimulus to that cell population. Alternatively, the basal level of SUAP expression can be generally determined for a certain cell type, and used as a reference for other populations of the same cell type. Assays suitable for detecting SUAP expression are well-known in the art, and include detection of SUAP RNA and/or protein levels by Northern or Western blot, for example as set forth in Examples 2 and 3 below.

The invention will now be illustrated by the following non-limiting examples.

Example 1 - Isolation and Characterization of SUAP Nucleotide and Amino Acid Sequences

Description of 32Dcl3 Cells - The murine hematopoietic cell line 32Dcl3 is a nontumorigenic, diploid cell line derived from normal mouse bone marrow. These cells strictly require IL-3 for continued growth. Culture conditions for 32Dcl3 cells are described in Valtieri M et al. (1987), *J. Immunol.*, 138, 3829-3835, the disclosure of which is herein incorporated by reference.

When 32Dcl3 cells are transferred to IL-3-free medium containing G-CSF, their cell number increases 4-5 fold, and after 12 days the entire population differentiates into morphologically normal, myeloperoxidase and lactoferrin positive metamyelocytes and granulocytes. Following terminal differentiation, the 32Dcl3 cells initiate apoptotic pathways which result in their death. Thus, IL-3 acts as an antagonist to terminal differentiation and as an inducer of proliferation.

Isolation of SUAP cDNA - Poly A+ mRNAs were derived from actively proliferating 32Dcl3 cells grown in the presence of IL-3, and from 32Dcl3 cells undergoing apoptosis as a result of IL-3 deprivation for 24 hours. These RNAs were subjected to representational difference analysis (RDA) according to the technique of Hubank M and Schatz DG (1994), *Nucl. Acids Res.* 22, 5640-5648, the disclosure of which is herein incorporated by reference.

The RDA analysis revealed a partial cDNA sequence which was isolated and used to screen a cDNA library derived from RNA extracted from 32Dcl3 cells at the later stages of G-CSF-induced terminal differentiation. The partial cDNA sequence predominantly hybridized to a transcript of approximately 1.1 kb. This transcript was cloned and sequenced by standard techniques, and revealed a coding region of 360 nucleotides encoding a peptide of 119 amino acids. The encoded protein has a predicted molecular weight of 13 kDa, but actually migrates as a doublet at ~15 kDa (see FIG. 3A). The amino acid sequence contained numerous lysine residues thought to be ubiquitination sites

(see FIG. 1), which is consistent with poor of the native peptide (see Example 3). Due to this peptide's relatively small size, numerous ubiquitination sites, and its association with the apoptotic response, it was named SUAP (for Small Ubiquitinated Apoptotic Protein). The nucleotide sequence of this isolated mouse SUAP cDNA is given in SEQ ID NO: 1, and the deduced amino acid sequence is given in FIG. 1 and SEQ ID NO: 2.

Example 2 - Multi-Tissue Expression of SUAP

A commercial multi-tissue Northern blot (Clontech) was probed with the ^{32}P-labeled SUAP cDNA sequence of SEQ ID NO: 1 under the following hybridization conditions: The filter was pre-hybridized under high stringency conditions at 42 °C in hybridization buffer (50% formamide; 5X SSC; 5X Denhardt's solution; 1% glycine; 50mM $NaPO_4$, pH 6.5; 250 μg/ml salmon sperm DNA) and hybridized overnight at 42 °C in hybridization buffer (50% formamide; 5XSSC; 1X Denhardt's solution; 100 mg/ml sodium dextran sulfate; 20mM $NaPO_4$, pH 6.5; 100 μg/ml salmon sperm DNA) containing the ^{32}P-labeled SUAP probe at a final concentration of 1 x 10^6 counts/ml. The filter was washed under high stringency conditions: 3X for 10 minutes each in 2XSSC/0.1% SDS at 42°C followed by 3X for 10 minutes each in 0.1XSSC/0.1% SDS at 42°C (see Mettus, RV et al. (1994), *Oncogene* 9: 3077-3086, the disclosure of which is herein incorporated by reference). Labeled RNA:probe complexes were visualized by phosphorimager analysis (Fuji) or by autoradiography. The results are presented in FIG. 2, and show that SUAP RNA is highly expressed in multiple tissues, including heart, brain, testis, liver and kidney. SUAP expression was also observed in the lung and spleen, albeit to a lesser extent.

Example 3 - SUAP Is a Target of the Proteosome

Construction of pSG5-SUAP/HA Plasmid Expressing Wild-Type SUAP with an HA tag - The entire SUAP cDNA of SEQ ID NO: 1 was fused in-frame with the HA-epitope tag (to facilitate detection by Western blot analysis), digested with BamHI and EcoRI, and sub-cloned into pSG5 expression vector

(Stratagene) that had been digested with BamHI and EcoRI. The nucleic acid sequence encoding the HA tag is:

5' TAC CCA TAC GAC GTC CCA GAC TAC GCT 3' (SEQ ID NO: 9)

The pSG5-SUAP/HA vector thus formed expressed SUAP tagged at the C-terminus with HA, and was used to transiently transfected into COS-7 cells.

Transient Transfection of COS-7 Cells - COS-7 cells were plated in 100mm dishes (50% confluency) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and penicillin-streptomycin. The following day, 5μg of plasmid DNA was added to DMEM supplemented with 10% NuSerum and 200μl of DEAE-Dextran solution (10mg/ml DEAE Dextran/2.5mM chloroquine; see Transient Expression of Proteins using COS-7 Cells, In: Current Protocols in Molecular Biology Vol.2 Unit 16.13.1 - 16.13.7, the disclosure of which is herein incorporated by reference). The cells were then incubated at 37°C for 3 hours, washed three times in PBS and re-fed with DMEM supplemented with 10% FBS and penicillin-streptomycin. Total RNA was isolated and SUAP RNA expression detected by Northern blot analysis as in Example 2, using radiolabeled SEQ ID NO: 1 as a probe. SUAP RNA was detected in the transfected COS-7 cells.

MG132 treatment of COS-7 cells transfected with pSG5-SUAP/HA - Although SUAP RNA was detected in the transfected COS-7 cells, significant SUAP expression was not detected at the protein level. To address the possibility that SUAP was a target of proteosome-mediated degradation, pSG5-SUAP/HA-transfected COS-7 cells were treated with different concentrations of the proteosome inhibitor MG132 (Affiniti Research Products, Ltd.) beginning at 24 hours post-transfection for varying lengths of time, according to the procedure of Lee DH and Goldberg AL (1998), *Trends in Cell Biol.* 8, 397-403, the disclosure of which is herein incorporated by reference. Concentrations and times for the MG132 treatment of COS-7 cells transfected with pSG5-SUAP/HA vector were as indicated in FIGS. 3A and 3B. COS-7 cells transfected with pSG5-SUAP/HA vector but not treated with MG132, and COS-

7 cells transfected with pSG5 vector and treated 2.5 μM MG132 for 6 hours, were used as controls.

Western blot analysis of transfected COS-7 cells was performed as follows. Cells were lysed in 1% NP-40/PBS, and the protein concentration of the lysate was quantitated using BioRad reagent (BioRad). Fifty micrograms of whole cell lysate was separated by 12% SDS-PAGE according to Laemmli UK (1970), *Nature* 227: 680 - 685, the disclosure of which is herein incorporated by reference. The resolved proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) in CAPS buffer (10mM CAPS, pH 11.0; 10% methanol) for 1 hour at room temperature. The filter was blocked in 3% non-fat dry milk/TBS-T (150mM NaCl; 20mM Tris-Cl, pH, 7.5; 0.05% Tween-20) for 1 hour at room temperature and incubated overnight at 4 °C in 3% non-fat dry milk/TBS (50mM NaCl; 20mM Tris-Cl, pH, 7.5) supplemented with an anti-HA antibody (Santa Cruz) at a concentration of 1:1000. The filter was washed three times with TBS-T for 10 minutes, and incubated in TBS-T supplemented with a 1:10,000 dilution of a donkey anti-rabbit secondary horse radish peroxidase (HRP) conjugated secondary antibody (Amersham/Pharmacia) at room temperature for 30 minutes. The filter was washed three times in TBS-T for 10 minutes, and the protein-antibody complexes visualized using Renaissance Reagent (Perkin Elmer).

FIG. 3A shows that SUAP, which migrates as a 15 kDa doublet, was stabilized in a dose- and time-dependent manner by the addition of MG132. Slower migrating forms of SUAP, which are believed to represent ubiquitinated SUAP, were observed in pSG5-SUAP/HA transfected COS-7 cells treated with 2.5μM MG132 for 16 hours, upon prolonged exposure of the autoradiogram from FIG. 9A (see FIG. 3B).

Lactacystin and clasto-*Lactacystin-ß-lactone treatment of COS-7 cells transfected with pSG5-SUAP/HA* - pSG5-SUAP/HA transfected COS-7 cells were also treated with Lactacystin and *clasto*-Lactacystin-ß-lactone, which are more specific inhibitors of the proteosome than MG132. (It is believed that *clasto*-Lactacystin-ß-lactone is a metabolite of Lactacystin and that the former is the actual species that interacts with the proteosome) All experimental cells

were treated for 16 hours with Lactacystin or *clasto*-Lactacystin-ß-lactone at the concentrations indicated in FIG. 3C. COS-7 cells transfected with pSG5-SUAP/HA vector but not treated with the proteosome inhibitors, and COS-7 cells transfected with pSG5 vector and treated with either 10 μM Lactacystin or 10 μM *clasto*-Lactacystin-ß-lactone for 16 hours, were used as controls. Protein extractions and Western blot analysis was performed as above.

As was observed in FIG. 3A for the MG132-treated cells, SUAP was stabilized in protein extracts of pSG5-SUAP/HA transfected COS-7 cells treated with Lactacystin and *clasto*-Lactacystin-ß-lactone in a dose dependent manner (FIG. 3B). The results of these studies demonstrate that endogenous SUAP is unstable and is subject to degradation by the proteosome.

Example 4 - SUAP Is Up-Regulated During G-CSF-Induced Terminal Differentiation of 32Dcl3 Cells

As discussed above in Example 1, it is known that granulocytic differentiation of 32Dcl3 cells is linked to apoptosis. Thus, SUAP RNA expression following exposure of 32Dcl3 cells to G-CSF was examined.

32Dcl3 cells were incubated in a G-CSF-containing medium for various periods of time as follows. Actively proliferating 32Dcl3 cells were washed and plated in IMDM supplemented with 10% fetal bovine serum (FBS) and 10% conditioned medium from a Chinese hamster ovary (CHO)-G-CSF cell line (as a source of G-CSF) for 0, 2, 4, 6, 8 and 10 days. Viability of the 32Dcl3 cells was monitored using trypan blue exclusion (FIG. 4A) and Cytospin preparations stained with Wright's Modified and Giemsa stains to show cytoplasmic and nuclear morphology (FIG. 4B).

RNA extracted from cells harvested at each time point was subjected to Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. This probe hybridizes to a transcript of ~1.1 kb representing SUAP mRNA.

As shown in FIG. 5, SUAP RNA is expressed at basal levels in 32Dcl3 cells grown in the presence of IL-3. Expression of SUAP RNA was induced in 32Dcl3 cells grown in the presence of G-CSF, and peaked at day 6. Although expression remained stable until day 8 (by which the majority of the 32Dcl3

population had terminally differentiated), SUAP expression declined by day 10. These results indicate that SUAP gene transcription is up-regulated during the G-CSF-induced terminal differentiation which precedes apoptosis of myeloid precursor cells.

Example 5 - SUAP Expression Is Up-Regulated During IL-3 Withdrawal-Induced Apoptotic Death of 32Dcl3 Cells

The role of SUAP expression during myeloid cell apoptosis was investigated in 32Dcl3 cells, which were induced to undergo apoptosis by removing IL-3 from the cell culture medium.

IL-3 Stimulates 32Dcl3 Cell Growth - 32Dcl3 cells were grown in the presence of IL-3, and their viability was monitored by trypan blue exclusion. As shown in FIG. 6A, these cells were still actively growing at eight days in culture.

Apoptosis in IL-3 Deprived 32Dcl3 Cells - Actively growing 32Dcl3 cells were washed and incubated in Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) supplemented with 10% FBS, but lacking IL-3. Viability of the cells was monitored by trypan blue exclusion. At approximately 1 day after withdrawal of IL-3, approximately half of the 32Dcl3 cells were dead. At five days post IL-3 withdrawal, virtually all the 32Dcl3 cells were dead. See FIG. 6B.

Apoptosis was directly demonstrated in IL-3 deprived 32Dcl3 cells as follows: Low molecular weight cellular DNA was extracted from IL-3 deprived 32Dcl3 cells by standard methods at 0, 6, 16 and 24 hour intervals following the removal of IL-3, and analyzed on a 1.8% agarose gel. FIG. 6C shows the appearance of low molecular weight DNA "ladders" at 16 and 24 hrs. post IL-3 withdrawal, which indicates the onset of apoptosis.

Induction of SUAP RNA During IL-3 Withdrawal-Induced Apoptosis of 32Dcl3 Cells - Actively growing 32Dcl3 cells were washed and incubated in IMDM supplemented with 10% FBS, but lacking IL-3. Viability of the cells was monitored by trypan blue exclusion. Total RNA was extracted from the IL-3 deprived 32Dcl3 cells at 0, 2, 4, 16 and 24 hours post IL-3 withdrawal, and

subjected to Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. The results are presented in FIG. 6D.

FIG. 6D shows that SUAP expression is induced in IL-3 deprived 32Dcl3 cells within 16 hours post IL-3 withdrawal. SUAP RNA levels remained elevated at 24 hours post-IL-3 removal, when approximately 50% of the 32Dcl3 cells were dead (see FIG. 6B). These results demonstrated that SUAP expression is specifically induced during the apoptotic death of 32Dcl3 cells.

Example 6 - SUAP Expression in Response to External Apoptotic Stimuli Is Blocked in 32D/*bcr-abl* Cells But Not in 32D/*v-abl* Cells.

32Dcl3 cells which have been transformed by plasmid vectors expressing the either the *v-abl* or *bcr-abl* oncogene can proliferate in the absence of IL-3. Incubation of 32D/*v-abl* cells in the presence of dimethyl sulfoxide (DMSO) blocks this proliferation and induces apoptosis. However, 32D/*bcr-abl* cells are resistant to DMSO-induced apoptosis. See FIG. 7A.

Construction of 32D/bcr-abl and 32D/v-abl Cells - The 32D/bcr-abl and 32D/v-abl cells were made according to Zhu J et al. (1996), *Blood* 87, 4368-4375, the disclosure of which is herein incorporated by reference.

Susceptibility or Resistance to DMSO-Mediated Apoptosis - To determine if the susceptibility or resistance to DMSO-mediated apoptosis is associated with SUAP expression, 32D/*v-abl* and 32D/*bcr-abl* cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and penicillin-streptomycin without DMSO ("0" days in DMSO), or in medium containing 1.5% DMSO for 1, 3, 5 or 7 days. Total RNA was isolated from each sample and subjected to Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. The results are presented in FIG. 7B, and show that SUAP RNA is expressed at basal levels in 32D/*v-abl* or 32D/*bcr-abl* cells in the absence of DMSO, despite the fact no IL-3 was provided to the cells.

However, SUAP expression is induced in 32D/*v-abl* cells between 48 and 72 hours following the addition of DMSO, which corresponds to the period when the cells begin to enter the apoptotic pathway (see FIG. 7A and Zhu J et al. (1996), *supra*). The levels of SUAP RNA expressed in the 32D/*v-abl* cells is

comparable to the levels of SUAP RNA induced in normal 32Dcl3 cells grown in the absence of IL-3 for 16-24 hours (see FIG. 6D). In contrast to 32D/*v-abl* cells, 32D/*bcr-abl* cells failed to up-regulate SUAP RNA, even after incubation for seven days in 1.5% DMSO (FIG. 7B).

Example 7 - Correlation of SUAP Expression and Apoptotic Death Induced by Chemotherapeutic Agents in 32Dcl3, 32D/*v-abl* and 32D/*bcr-abl* Cells

The relationship between apoptosis induced by anti-leukemic/chemotherapeutic agents and endogenous SUAP expression in normal 32Dcl3 myeloid cells, and in *v-abl* and *bcr-abl* transformed 32Dcl3 myeloid cells is investigated as follows.

Determination of Optimal Apoptosis-Inducing Concentrations of Chemotherapeutic Agents - $4x10^5$ normal 32Dcl3 cells (in the presence of IL-3) as well as v-*abl* and *bcr-abl*-transformed 32Dcl3 cell lines (in the absence of IL-3) in logarithmic growth phase are treated with various chemotherapeutic agents at concentrations ranging from 1nM to 100μM, in order to determine the optimal concentration at which each agent induces apoptosis. The chemotherapeutic agents are: STI571 (a specific tyrosine kinase inhibitor of the BCR-ABL oncoprotein), hydroxyurea (HU, an inhibitor of ribonucleotide reductase), cytosine arabinoside (Ara-C), methotrexate (an inhibitor of thymidelate synthetase), etoposide (an inhibitor of topoisomerase II), and vincristine (which inhibits the formation of microtubules).

The cells are analyzed daily for survival and induction of the apoptotic response over a period of 10 days as follows: Cell number and viability is determined by trypan blue exclusion. Cell cycle distribution and the presence of sub-diploid cells in the treatment groups is determined by flow cytometry analysis. DNA is isolated from the cells and electrophoresed on 1.8% agarose gels to demonstrate the appearance of "ladders," which result from the endonucleolytic cleavage of DNA characteristic of apoptosing cells.

For the flow cytometric analysis, cells are washed twice in 1% phosphate-buffered saline (PBS) containing 1% FBS, and the washed cells are fixed in 80% cold ethanol for 60 minutes, pelleted and resuspended in PBS/1%

FBS containing 50μg/ml of propidium iodide and 1 mg/ml of RNAse. After a 30 minute incubation at 37° C, the cells are analyzed with a Coulter Epic Elite flow cytometer.

Expression of SUAP RNA - 32Dcl3, 32D/v-*abl* and 32D/*bcr-abl*-cells are treated with the optimal apoptosis-inducing concentration of each drug, as determined above. Total RNA is isolated from each treatment group and subjected to Northern blot analysis as in Example 2, using radiolabel SUAP cDNA as a probe.

Expression of SUAP Protein - 32Dcl3, 32D/v-*abl* and 32D/*bcr-abl*-cells are treated with the optimal apoptosis-inducing concentration of each drug as determined above. Each treatment group is divided in two; one group is treated with 2.5μM MG132 for a period of 2 hours prior to protein extraction, and the other is not. Protein extracts from all groups are prepared and subjected to Western blot analysis as in Example 3. Endogenous SUAP is detected by a rabbit polyclonal antiserum raised against the full length SUAP protein.

Example 8 - Ectopic Expression of SUAP Suppresses the Proliferation of 32Dcl3 Cells in Response to IL-3

Construction of 32Dcl3 Cells that Inducibly Express SUAP - An inducible expression construct that places SUAP expression under the control of an isopropyl-thio-ß-D-galactopyranoside (IPTG)-inducible promoter was constructed with the SUAP cDNA sequence of SEQ ID NO: 1 and the LacSwitch system from Stratagene. This construct and a vector which encoded the repressor of the IPTG-inducible promoter were electroporated into 32Dcl3 cells using a GenePulser (BioRad) set at 300 volts and a capacitance of 960 μFaradays. The electroporated cells were incubated in IMDM medium supplemented with 10% FBS and IL-3 for 72 hrs. Stable cell lines were selected using IMDM medium supplemented with 10% FBS, IL-3, hygromycin (1 mg/ml) and geneticin (G418; 0.5 mg/ml). The medium was changed at 3-4 day intervals until drug-resistant colonies emerged. Clonal cells from the drug resistant colonies were expanded by serial dilution in 96-well microtiter plates as described in Patel G et al., (1993), Mol. Cell. Biol. 13, 2269-2276, the disclosure of which is herein incorporated by reference. All single cell clones

were expanded and monitored for the expression of SUAP following the addition of IPTG, using standard Northern and Western blot analyses as described in Examples 2 and 3, respectively.

Three 32Dcl3 clones that expressed high levels of SUAP upon treatment with IPTG were isolated. SUAP expression in these clones was confirmed as follows: 32Dcl3/SUAP and control cells were grown in medium containing 10% WEHI-3B cultured supernatant (as a source of IL-3) in the presence and absence of 5mM IPTG for 24 hours. Total RNA was extracted from these cells and subjected to Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. FIG. 8A shows that SUAP expression in induced in all three clones in the presence of IPTG. No significant SUAP expression was seen in 32Dcl3 control cells transfected with vector alone and treated with IPTG, or in the clones grown in the absence of IPTG. Similar expression profiles were obtained by Western blot analysis of protein extracts from the three clones and from control cells, all of which were treated with 2.5μM MG132 for 2 hours prior to protein extraction.

Effect of Ectopic SUAP Expression on IL-3 Mediated Proliferation of 32Dcl3 Cells - The 32Dcl3/SUAP clones described above and the vector-only transfected 32Dcl3 control cells were seeded at a density of $2x10^5$ cells/ml in medium containing 10% WEHI-3B cultured supernatant (as a source of IL-3), in the presence and absence of 5mM IPTG. Aliquots of cells were removed at 24 hour intervals, and their number and viability determined by trypan blue exclusion. FIG. 8B shows that the control cells proliferated normally in response to IL-3, but that the three SUAP-transfected cell lines exhibited suppressed rates of proliferation. Similar growth arrest was observed during G-CSF-mediated terminal differentiation of these cell lines, although no changes in their cytoplasmic and nuclear morphologies was observed. These results indicate that the expression of SUAP is correlated with the growth arrest that often precedes the onset of apoptosis in myeloid cells.

Example 9 - Correlation of Ectopic Expression of SUAP and the Susceptibility of 32Dcl3, 32/V-*abl* and 32D/*bcr-abl* Cells to Apoptotic Death Induced by Chemotherapeutic Agents

The ability of ectopically expressed SUAP to enhance the activity of external apoptosis-inducing stimuli in 32D/v-*abl* and 32D/*bcr-abl* cells is investigated as follows.

Determination of Optimal Apoptosis-Inducing Concentration of Chemotherapeutic Agents - 32D/*v-abl* and 32D/*bcr-abl* cell lines that express SUAP in an IPTG-inducible manner are generated using the LacSwitch system (Stratagene) as described above for normal 32Dcl3 cells. 32Dcl3, 32D/*v-abl* and 32D/*bcr-abl* clones that express high levels of SUAP are treated with hydroxyurea (HU), cytarabine (Ara-C), methotrexate, etoposide, and vincristine at concentrations ranging from 1nM to 100μM, in order to determine the optimal concentration at which each agent induces apoptosis. The cells are analyzed daily for survival and induction of the apoptotic response over a period of 10 days, as in Example 7.

Expression of SUAP RNA - 32Dcl3, 32D/v-*abl* and 32D/*bcr-abl*-cells are treated with the optimal apoptosis-inducing concentration of each drug, as determined above. Total RNA is isolated from each treatment group and subjected to Northern blot analysis as in Example 2, using radiolabel SUAP cDNA as a probe.

Expression of SUAP Protein - 32Dcl3, 32D/v-*abl* and 32D/*bcr-abl*-cells are treated with the optimal apoptosis-inducing concentration of each drug as determined above. Each treatment group is divided in two; one group is treated with 2.5μM MG132 for a period of 2 hours prior to protein extraction, and the other is not. Protein extracts from all groups are prepared and subjected to Western blot analysis as in Example 3. Endogenous SUAP is detected by a rabbit polyclonal antiserum raised against the full length SUAP protein.

Example 10 - SUAP Expression Is Up-Regulated During the Apoptosis of Breast Tumor Cells

Apoptosis Induced by Serum Starvation- MCF7 breast carcinoma cells were induced to undergo apoptosis by removal of serum from the culture medium, which deprived the cells of factors normally needed to suppress the apoptotic response. Total RNA was extracted from serum-starved MCF7 cells at 0, 24, 48 and 72 hours and subjected to Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. The results are shown in FIG. 9A, which reveals that SUAP expression is up-regulated in serum-starved MCF7 cells at 24, 48 and 72 hours.

Apoptosis Induced by Anticancer Drugs - SUAP expression was also upregulated in MCF7 cells in response to treatment of the cells with the anticancer drugs taxol (paclitaxel), etoposide, cisplatin, and camptothecin, all of which induce apoptosis in the cancer cell.

MCF7 cells were grown in the presence of taxol (5nM), etoposide (1μM), cisplatin (15μM), or camptothecin (40nM), and total RNA was extracted from the cells at 0, 24 and 48 hours (for the taxol-treated cells), or at 0, 24, 48 and 72 hours and (for the etoposide, cisplatin and camptothecin-treated cells). The RNA preparations were analyzed by Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. The results are shown in FIGS. 9B through 9E.

The results show that SUAP was expressed at basal levels in untreated, actively proliferating MCF7 cells (see the "0" hour timepoint in FIGS. 9B through 9E). The effect of each drug on SUAP expression in MCF7 cells varied slightly: FIG. 9B shows that SUAP expression was dramatically induced by taxol within 48 hours. FIG. 9C shows that etoposide induces SUAP at 48 and 72 hours. FIGS. 4D and 9E show, respectively, that cisplatin and camptothecin induce SUAP at 24, 48 and 72 hours.

Taken together, these results indicate that SUAP mediates the apoptotic response of cells induced by anti-cancer drugs, and also shows that detection of SUAP expression is a marker of apoptosis in cells treated with anti-cancer drugs.

Example 11 - Correlation of SUAP Expression and Apoptotic Death Induced by Chemotherapeutic Agents in Breast Tumor Cells

The relationship between apoptosis induced by chemotherapeutic agents and endogenous SUAP expression in breast cancer cells is investigated as follows.

Determination of Optimal Apoptosis-Inducing Concentration of Chemotherapeutic Agents - Ten different breast carcinoma cell lines are seeded at a density of 1 x 10^4 cells/60 mm dish in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and penicillin-streptomycin. Chemotherapeutic agents are added 24 hours post-plating at different concentrations, and treatment is continued over a 4-7 day period to determine the optimal concentration at which these compounds induce apoptosis in each cell line. The chemotherapeutic agents are taxol, etoposide, camptothecin, cisplatin, doxorubicin and 5-fluorouracil (5FU). All of these agents are available from Calbiochem, along with information concerning appropriate ED_{50} concentrations and solubilities.

The cell lines are: T47D, MCF7, BT474, 361 and ZR-75-30 (which are estrogen-receptor positive); and 435, HTB126, 231, 435 and SKBR3 (which are estrogen-receptor negative). These cell lines are described in Cappelletti V et al. (1993), *Breast Cancer Res.* 26, 275-281 and Chen X et al. (2000), *J. Natl. Cancer Inst.* 92, 1403-1413, the entire disclosures of which are herein incorporated by reference. Estrogen receptor (ER)-negative tumors typically exhibit a more aggressive phenotype and are not well differentiated as compared to estrogen receptor-positive tumors (see Ciocca DR and Eledge R (2000), *Endocrine* 13, 1-10).

Cell number and viability in each treatment group is determined by trypan blue exclusion. Apoptosis in each treatment group is directly demonstrated by flow cytometry and DNA analysis as in Example 7 above.

Expression of SUAP RNA - The breast carcinoma cells are treated with the optimal apoptosis-inducing concentration of each drug as determined above. Total RNA is isolated from each treatment group and subjected to Northern blot analysis as in Example 2, using radiolabel SUAP cDNA as a probe.

Expression of SUAP Protein - The breast carcinoma cells are treated with the optimal apoptosis-inducing concentration of each drug as determined above. Each treatment group is divided in two; one group is treated with 2.5μM MG132 for a period of 2 hours prior to protein extraction, and the other is not. Protein extracts from all groups are prepared and subjected to Western blot analysis as in Example 3. Endogenous SUAP is detected by a rabbit polyclonal antiserum raised against the full length SUAP protein.

Example 12 - Correlation of Ectopic Expression of SUAP and Apoptosis in Breast Cancer Cells

The effect of ectopic SUAP expression on apoptosis in breast tumor cells is investigated as follows.

Construction of SUAP-Expressing Breast Cancer Cell Clones - Estrogen receptor-positive and estrogen receptor-negative breast carcinoma cell lines are transfected with an IPTG-inducible SUAP expression vector as in Example 8 above. Single cell clones expressing high levels of SUAP, as confirmed by Northern and/or Western blot analysis (see Example 8), are isolated and propagated in growth medium in the presence or absence of 5mM IPTG. Control cells transfected with vector alone, or not transfected at all, are subjected to the same treatments as the SUAP-expressing clones.

Assessment of Apoptosis Induced by SUAP Expression - Induction of apoptosis in experimental and control cells is assessed by cell viability and DNA fragmentation assays as described in Example 7, at daily intervals over a 7 day period.

Example 13 - Correlation of Ectopic expression of SUAP and Susceptibility of Breast Cancer Cells to Apoptotic Death Induced by Chemotherapeutic Agents

The ability of ectopically expressed SUAP to enhance the activity of external apoptosis-inducing stimuli in breast cancer cells is investigated as follows.

Susceptibility to Apoptosis Induced by Chemotherapeutic Agents- The SUAP expressing clones and vector-only transfected control cells of Example 12 are treated with taxol, etoposide, camptothecin, cisplatin, doxorubicin and

5FU in the presence or absence of 5mM IPTG. Induction of apoptosis in experimental and control cells is assessed by cell viability and DNA fragmentation assays as described in Example 7, at daily intervals over a 7 day period following the addition of the chemotherapeutic agents to the culture medium.

Example 14 - SUAP Expression Is Up-Regulated During the Apoptosis of Prostate Tumor Cells

SUAP Expression in Irradiated Prostate Tumor Cells - Androgen-independent DU145 and androgen-responsive LnCap prostate tumor cells grown in androgen-containing growth media (Roswell Park Memorial Institute Media [RPMI; Invitrogen] with 10% FBS) were exposed to 15 Gy ionizing (gamma) radiation. This radiation dose induces apoptotic death by damaging the cellular DNA.

Total RNA was extracted from the irradiated DU145 and LnCap cells at 0, 24, 48 and 72 hours, and subjected to Northern blot analysis as in Example 2 using radiolabeled SUAP cDNA as a probe. The results, presented in FIG. 10A, show that gamma-irradiation induces the expression of SUAP in the androgen-independent DU145 prostate tumor cells. However, SUAP expression was not observed in the androgen responsive LnCap prostate tumor cells. These results indicate that apoptosis in irradiated LnCap cells is blocked by androgen-mediated suppression of SUAP expression.

SUAP Expression in Androgen Ablated LnCap Cells - Actively proliferating LnCap cells were washed once in RPMI, and re-plated in RPMI supplemented with 10% charcoal-stripped FBS. Charcoal-stripping the FBS removes androgens necessary for suppression of apoptosis in the cells.

Total RNA was extracted from the androgen-ablated LnCap cells at 0, 24, 48 and 72 hours, and subjected to Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. The results, given in FIG. 10B, show that androgen ablation up-regulates SUAP expression in LnCap cells after 72 hours growth in androgen-free media.

SUAP Expression Is Up-Regulated in Androgen-Ablated LnCap Cells by Radiation - Actively proliferating LnCap cells were washed once in RPMI, re-

plated in RPMI supplemented with 10% charcoal-stripped FBS and exposed to 15 Gy ionizing (gamma) radiation. Total RNA was isolated from the irradiated cells at 0, 24, 48 and 72 hours, and subjected to Northern blot analysis as in Example 2, using radiolabeled SUAP cDNA as a probe. The results, presented in FIG. 10C, show that SUAP was expressed in the androgen-ablated LnCap cells at 24, 48 and 72 hours post irradiation.

Taken together, these results indicate that SUAP is induced in prostate tumor cells in response to apoptotic stimuli such as irradiation and androgen ablation. These results also show that detection of SUAP expression in prostate cancer cells is a marker of apoptosis.

Example 15 - Correlation of Endogenous SUAP Expression with Apoptotic Death Induced by Chemotherapeutic Agents in Prostate Tumor Cells

The relationship between apoptosis induced by chemotherapeutic agents and endogenous SUAP expression in prostate tumor cells in investigated as follows.

Determination of Optimal Apoptosis-Inducing Concentration of Chemotherapeutic Agents - Actively proliferating DU145 and LnCap cells grown in androgen-containing growth medium (RPMI with 10% FBS) are treated with taxol, cisplatin, etoposide, estramustine, and the combinations taxol/estramustine and taxol/estramustine/etoposide at different concentrations over a 4 - 7 day period, to determine the optimal concentration at which these agents induce apoptosis in each cell line. Cell number and viability in each treatment group is determined by trypan blue exclusion. Apoptosis in each treatment group is directly demonstrated by cell viability and DNA fragmentation assays as in Example 7.

Expression of SUAP RNA - DU145 and LnCap cells are grown in androgen-containing growth medium (RPMI with 10% FBS) are treated with the optimal apoptosis-inducing concentration of each drug or drug combination determined above. Total RNA is isolated from each treatment group and subjected to Northern blot analysis as in Example 2, using radiolabel SUAP cDNA as a probe.

Expression of SUAP Protein - DU145 and LnCap cells are grown in androgen-containing growth medium (RPMI with 10% FBS), and are treated with the optimal apoptosis-inducing concentration of each agent as determined above. Each treatment group is divided in two; one group is treated with 2.5μM MG132 for a period of 2 hours prior to protein extraction, and the other is not. Protein extracts from all groups are prepared and subjected to Western blot analysis as in Example 3. Endogenous SUAP is detected by a rabbit polyclonal antiserum raised against the full length SUAP protein.

Example 16 - Correlation of Ectopic Expression of SUAP and Apoptosis in Prostate Tumor Cells

The effect of ectopic SUAP expression on apoptosis in prostate tumor cells is investigated as follows.

Construction of SUAP-Expressing Prostate Tumor Cell Clones - DU145 and LnCap prostate tumor cells are transfected with an IPTG-inducible SUAP expression vector as in Example 8. Single cell clones expressing high levels of SUAP, as confirmed by Northern and/or Western blot analysis as in Example 8, are isolated. Each clone is propagated in androgen-containing medium in the presence or absence of 5mM IPTG. Control DU145 and LnCap cells transfected with vector alone were subjected to the same treatments as the SUAP-expressing clones.

Assessment of Apoptosis Induced by SUAP Expression - Induction of apoptosis in experimental and control cells is assessed by cell viability and DNA fragmentation assays as described in Example 7, at daily intervals over a 7 day period.

Example 17 - Ectopic Expression of SUAP and Susceptibility of Prostate Tumor Cells to Apoptotic Death Induced by Chemotherapeutic Agents, Radiation And Hormone Ablation

The ability of ectopically expressed SUAP to enhance the activity of external apoptosis-inducing stimuli in prostate tumor cells is investigated as follows.

Susceptibility to Apoptosis Induced by Chemotherapeutic Agents- The SUAP expressing DU145 and LnCap clones and vector-only transfected control

cells of Example 16 are propagated in androgen-containing medium and treated with taxol, etoposide, cisplatin or estramustine in the presence or absence of 5mM IPTG. Induction of apoptosis in experimental and control cells is assessed by cell viability and DNA fragmentation assays as described in Example 7, at daily intervals over a 7 day period following the addition of the drug to the culture medium.

Susceptibility to Apoptosis Induced by Radiation or Androgen Ablation - The SUAP-expressing DU145 clones and vector-only transfected DU145 control cells of Example 16 are either exposed to 15 Gy ionizing (gamma) radiation, or grown in medium containing 10% charcoal-stripped FBS, in the presence or absence of 5mM IPTG. The SUAP-expressing LnCap clones and vector-only transfected LnCap control cells of Example 16 are grown in medium containing 10% charcoal-stripped FBS and exposed to 15 Gy ionizing (gamma) radiation, in the presence and absence of 5mM IPTG. Induction of apoptosis in experimental and control cells is assessed by cell viability and DNA fragmentation assays as described in Example 7, at daily intervals over a 7 day period following radiation treatment and/or androgen ablation.

Example 18 - Construction and Expression of SUAP Derivatives

Two SUAP derivatives were made and expressed in COS-7 cells, to determine the effect of the mutations on SUAP stability inside the cell. The first derivative was a "lysine to arginine" (K→R) derivative of SUAP, in which each of the lysine residues at positions 6, 8, 39, 83, 89, 99, 101, 104 and 114 were converted to arginines. The second derivative was a Myc/SUAP fusion protein, in which a 12-amino acid epitope from Myc was inserted into the SUAP protein immediately after the initial methionine. Each derivative also had an HA epitope tag on the C-terminal end.

Construction of pSG5-SUAP/HA Plasmid Expressing Wild-Type SUAP with an HA tag - pSG5-SUAP/HA plasmid was constructed as above for Example 3.

Construction of pSG5-Myc/SUAP/HA Plasmid Expressing the Myc/SUAP Fusion Protein Derivative Tagged with HA - Using oligonucleotide-directed fusion PCR, the nucleotide sequence encoding the Myc epitope was

fused into the SUAP cDNA of SEQ ID NO: 1 immediately after the first codon (the ATG at nucleotide numbers 193-195). The nucleotide sequence encoding the Myc epitope was:

5' GCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTG 3' (SEQ ID NO: 10).

The nucleotide sequence of the nucleic acid encoding the Myc/SUAP fusion protein is given in SEQ ID NO: 5. The amino acid sequence encoded by SEQ ID NO: 5 is given in SEQ ID NO: 6. To produce the Myc/SUAP/HA derivative, the cDNA of SEQ ID NO: 5 was fused in-frame with the nucleic acid encoding the HA-epitope tag (SEQ ID NO: 9), digested with BamHI and EcoRI, and sub-cloned into pSG5 vector that had been digested with BamHI and EcoRI. The resulting plasmid expressed the Myc/SUAP/HA fusion protein.

Construction of pSG5-K→R SUAP/HA Plasmid Encoding the K→R SUAP Derivative Tagged with HA - All lysines in the wild-type SUAP amino acid sequence were changed to arginines by oligonucleotide-directed mutagenesis of the cDNA of SEQ ID NO: 1, according to the procedure of Zaret KS et al. (1990), *Proc. Natl. Acad. Sci. USA* 87: 5469-5473, the entire disclosure of which is herein incorporated by reference.

Briefly, four primers for each mutation or set of mutations were constructed: two complimentary internal primers, each of which contained the desired mutation, and two external primers, which contained suitable cloning restriction sites and were complimentary to one end of either DNA strand. Two individual PCR reactions were then performed per mutation or per set of mutations, using one external and one mutated internal primer. The products of both PCR reactions were gel purified and combined to create a template for a third PCR reaction using the two external primers. The final PCR product (containing the desired mutation) was gel purified, digested with the appropriate restriction enzymes and sub-cloned into a pSG5 parental vector that had been digested with the identical restriction enzymes. The structure and integrity of each mutant was confirmed by standard Sanger dideoxy sequence analysis.

The sequences of the internal primers that contained the K→R mutations were as follows (sense primer only; the anti-sense primer was complimentary to

the sense primer). Certain primers contained multiple mutations as part of the same primer. The mutated codons are underlined:

Lysines 6 and 8
5' CGGATTTCAAGGCCCAGGACGTTTCAG 3' (SEQ ID NO: 11)

Lysine 39
5' CTCATCTCCAGGTCCTTCCAG 3' (SEQ ID NO: 12)

Lysines 83 and 89
5' GAGAACTGCAGGACCACTTTGGGCTGGAGATATGAGCAA 3' (SEQ ID NO: 13)

Lysines 99, 101, 104, and 114
5'AGCAGCCAGAGGTACAGAGAGGGGAGATACATCATTGAACTCAAC CACATGATCAGAGACAACGGC 3' (SEQ ID NO: 14)

The external primers used in each mutation reaction were the same, and their sequences were as follows:

Primer 1 (5'): 5' CCCGCGTGGGGCCACTGCCCT 3' (SEQ ID NO: 15)

Primer 2 (3'): 5' CTTCCCCTGTGGGGGCCCTGA 3' (SEQ ID NO: 16)

The amino acid sequence of the K→R SUAP derivative encoded by the final K→R mutant cDNA is given in SEQ ID NO: 7. To produce the K→R SUAP/HA derivative, the final K→R mutant cDNA was fused in-frame with the HA-epitope tag (SEQ ID NO:9), digested with BamHI and EcoRI, and sub-cloned into pSG5 vector that had been digested with BamHI and EcoRI to form pSG5-K→R SUAP/HA. The resultant plasmid expressed the K→R SUAP/HA derivative.

Transfection of COS-7 Cells - COS-7 cells were transiently transfected with the plasmids expressing wild-type SUAP and the SUAP derivatives described above, as in Example 3.

Half-Lives of Wild-Type SUAP and SUAP Derivatives in COS-7 Cells - COS-7 cells were transiently transfected with 5μg of pSG5-SUAP/HA, pSG5-K→R SUAP/HA or pSG5-Myc SUAP/HA as in Example 3. At 48 hrs. post-transfection, the cells were starved in methionine-, cysteine- and leucine-free DMEM supplemented with 10% FBS for 1 hour at 37 °C. The cells were then

stimulated to begin translation upon the addition of ^{35}S Express Protein Translabel (containing methionine, leucine and cysteine) at a concentration of 250μCi/ml in the presence of 20μM MG132. After 1 hour, the cells were washed free of the radioactive label and "chased" with DMEM containing all amino acids, and supplemented with 10% FBS and 20μM MG132. The cells were harvested at 0, 1, 2 and 3 hours post DMEM "chase" and lysed in 1% NP-40/PBS supplemented with 1mM PMSF; 2μg/ml pepstatin; 2μg/ml leupeptin; 2μg/ml aprotinin; 1mM NaF; 1mM Na_3VO_4. Three hundred micrograms of this lysate was immunoprecipitated with an anti-HA antibody and protein G Sepharose for 1 hour at 4 °C. The immunoprecipitates were washed three times in PBS and the beads resuspended in Laemmli buffer (see Example 3). Proteins were resolved by 12% SDS-PAGE. After electrophoresis, the gel was fixed in 10% glacial acetic acid/30% methanol, enhanced with "Enhance" solution from Perkin Elmer and dried. The results are given in FIG. 11A. Protein bands from the autoradiogram shown in FIG. 11A were quantitated using the MacBas program in conjunction with a Fuji Phosphorimager, and plotted in FIG. 11B as % of protein from the 0 hour timepoint remaining at 1, 2 or 3 hours. As shown in FIG. 11B, the Myc/SUAP/HA fusion protein was slightly more stable than the wild-type SUAP/HA, and the K→R SUAP/HA mutant had a significantly longer half-life in COS-7 cells than either the wild-type SUAP/HA or Myc/SUAP/HA fusion proteins.

All documents referred to herein are incorporated by reference. While the present invention has been described in connection with the preferred embodiments of the various figures, it is understood that other similar embodiments may be used or modifications and additions made to the described embodiments for performing the same function of the present invention without deviating therefrom. Therefore, the present invention should not be limited to any single embodiment, but rather should be construed in breadth and scope in accordance with the recitation of the appended claims.

We claim:

1. A method of inducing growth arrest or apoptosis in cells, comprising contacting the cells with an effective amount of a compound comprising SUAP or biologically active derivative, homolog or analog thereof, such that the compound is introduced into the cell and growth arrest or apoptosis is effected.

2. The method of claim 1, wherein the compound comprises a peptide selected from the group consisting of SEQ ID NO: 2; SEQ ID NO: 4; SEQ ID NO: 6; and SEQ ID NO: 7.

3. The method of claim 1, wherein contacting the cells with the compound comprises transfecting the cell with a nucleic acid sequence encoding the compound.

4. The method of claim 3, wherein the nucleic acid sequence comprises a plasmid expression vector.

5. The method of claim 3, wherein the nucleic acid sequence comprises a nucleic acid selected from the group consisting of SEQ ID NO:1; SEQ ID NO: 3; and SEQ ID NO: 5.

6. The method of claim 1, wherein contacting the cells with the compound comprises the direct introduction of the compound into the cell.

7. The method of claim 6, wherein the compounds are directly introduced into the cell by liposomal transfer or protein transduction.

8. The method of claim 6, wherein the compound comprises a modification that directs entry of the compound into the cell.

9. The method of claim 8, wherein the modification comprises a protein transduction domain.

10. The method of claim 9, wherein the protein transduction domain comprises SEQ ID NO: 8.

11. The method of claim 1 wherein the cells comprise cells that are resistant to apoptosis.

12. The method of claim 1, wherein the cells comprise cancer cells.

13. The method of claim 12, wherein the cancer cells are from a cancer selected from the group consisting of sarcoma; melanoma; carcinoma; adenocarcinoma; glioma; glioblastoma; astrocytoma; and hematological neoplasia.

14. The method of claim 13, wherein the hematological neoplasia is acute lymphoblastic leukemia, chronic lymphocytic leukemia, or chronic myelocytic leukemia.

15. The method of claim 13, wherein the cancer originates in a tissue selected from the group consisting of breast; ureter; bladder; prostate; testis; ovary; cervix; uterus; vagina; lung; stomach; large intestine; small intestine; colon; rectum; pancreas; adrenal glands; mouth; esophagus; brain; spinal cord; kidney; liver; gall bladder; lymphatic system; smooth muscle; striated muscle; bone; bone marrow; skin; and tissues of the eye.

16. The method of claim 12, wherein the cancer is in any prognostic stage of development.

17. The method of claim 1, wherein the cells comprise cells from a non-cancerous proliferative disorder.

18. The method of claim 17, wherein the non-cancerous proliferative disorder is selected from the group consisting of hemangiomatosis in the newborn; secondary progressive multiple sclerosis; chronic progressive myelodegenerative disease; neurofibromatosis; ganglioneuromatosis; keloid formation; Paget's Disease of the bone; fibrocystic disease; sarcoidosis; Peronies fibrosis; Duputren's fibrosis, cirrhosis, atherosclerosis; and vascular restenosis.

19. A method of enhancing the effect of an external apoptotic stimulus on cells, comprising the steps of:

(1) contacting the cells with an effective amount of a compound comprising SUAP or biologically active derivative, homolog or analog thereof, such that the compound is introduced into the cell; and

(2) applying an external apoptotic stimulus to the cells.

20. The method of claim 19, wherein the compound comprises a peptide selected from the group consisting of SEQ ID NO: 2; SEQ ID NO: 4; SEQ ID NO: 6; and SEQ ID NO: 7.

21. The method of claim 19, wherein contacting the cells with the compound comprises transfecting the cell with a nucleic acid sequence encoding the compound.

22. The method of claim 21, wherein the nucleic acid sequence comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1; SEQ ID NO: 3; and SEQ ID NO: 5.

23. The method of claim 19, wherein the compound comprises a modification that directs entry of the compound into the cell.

24. The method of claim 19 wherein the cells comprise cells that are resistant to apoptosis.

25. The method of claim 19, wherein the cells comprise cancer cells.

26. The method of claim 19, wherein the cells comprise cells from a non-cancerous proliferative disorder.

27. The method of claim 19, wherein the external apoptosis inducing-stimulus is selected from the group consisting of ionizing radiation; a chemotherapeutic; and deprivation of agents which prevent apoptosis.

28. The method of claim 19, wherein the external apoptosis inducing-stimulus is STI571.

29. The method of claim 19, wherein the cells are contacted with the compound prior to administration of the external apoptosis inducing-stimulus.

30. The method of claim 29, wherein the cells are contacted with the compound about 24 hours prior to administration of the external apoptosis inducing-stimulus.

31. The method of claim 29, wherein the cells are contacted with the compound about 18 hours prior to administration of the external apoptosis inducing-stimulus.

32. The method of claim 29, wherein the cells are contacted with the compound about 6 to 12 hours prior to administration of the external apoptosis inducing-stimulus.

33. The method of claim 19, wherein the cells are contacted with the compound simultaneously with administration of the external apoptosis inducing-stimulus.

34. The method of claim 19, wherein the cells are contacted with the compound after administration of the external apoptosis inducing-stimulus.

35. The method of claim 34, wherein the cells are contacted with the compound about 1 to 6 hours after administration of the external apoptosis inducing-stimulus.

36. A method of identifying external stimuli which induce growth arrest or apoptosis in cells, comprising the steps of:

(1) determining the basal level of endogenous SUAP expression in the cells;

(2) administering an external stimulus to the cells; and

(3) determining the level of endogenous SUAP expression in the cell after administration of the external stimulus;

wherein an increased level of endogenous SUAP expression in the cells relative to the basal level indicates that the external stimulus has induced growth arrest or apoptosis in the cell.

37. The method of claim 36 wherein the external stimulus comprises radiation or a chemical compound.

38. An isolated nucleic acid sequence comprising:

(a) SEQ ID NO: 3;

(b) a nucleic acid sequence complementary to (a);

(c) a nucleic acid having at least 96% sequence identity with (a) or (b); or

(d) a nucleic acid sequence that hybridizes to (a) or (b) in 7% sodium dodecyl sulfate, 0.5 M $NaPO_4$, 1 mM EDTA at 50 °C with washing in 2XSSC, 0.1% sodium dodecyl sulfate at 50 °C.

39. The nucleic acid of claim 38, wherein (c) or (d) encodes a protein that:

(i) induces growth arrest or apoptosis in cells; or

(ii) comprises at least one epitope of SUAP.

40. The nucleic acid of claim 38, wherein (c) has at least 98% sequence identity with (a) or (b).

41. A compound comprising SEQ ID NO: 6.

42. A compound comprising SEQ ID NO: 7.

43. An antibody that specifically binds to an epitope on the compound of claim 41, but does not bind to an epitope of SEQ ID NO: 2.

44. The antibody of claim 43 which is a polyclonal antibody.

45. The antibody of claim 43 which is a monoclonal antibody.

46. A hybridoma producing the monoclonal antibody of claim 45.

47. An antibody that specifically binds to an epitope on the compound of claim 42, but does not bind to an epitope of SEQ ID NO: 2.

48. The antibody of claim 47 which is a polyclonal antibody.

49. The antibody of claim 47 which is a monoclonal antibody.

50. A hybridoma producing the monoclonal antibody of claim 50.

MVRISKPKTF QAYLDDCHRR YSCAHCRAHL ANHDDLISKSF QGSQGRAYLF NSVVNVGCG PAEERVLLTGL HAVADIHCEC KTTLGWKYEQ AFESSQKYKE GKYIIELNHM IKDNGWD*

FIG. 1

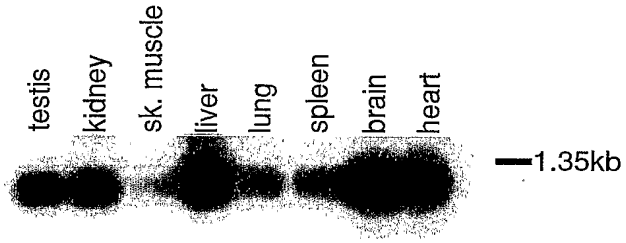

testis
kidney
sk. muscle
liver
lung
spleen
brain
heart
1.35kb

FIG. 2

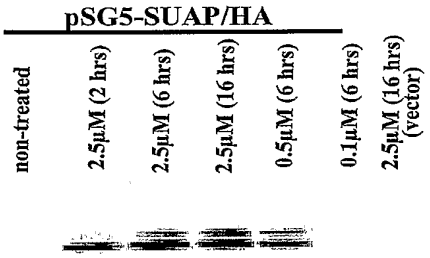


FIG. 3A

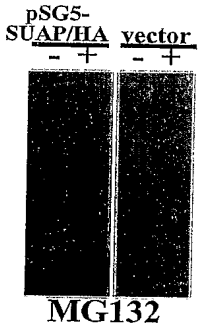


FIG. 3B

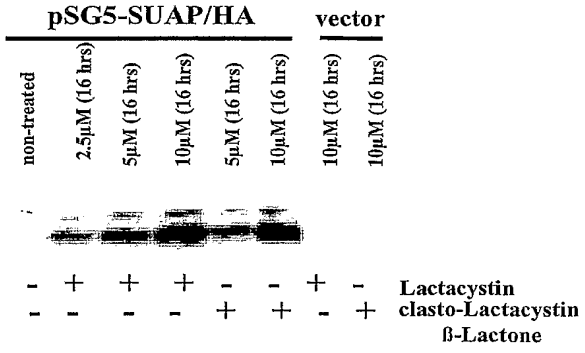


FIG. 3C

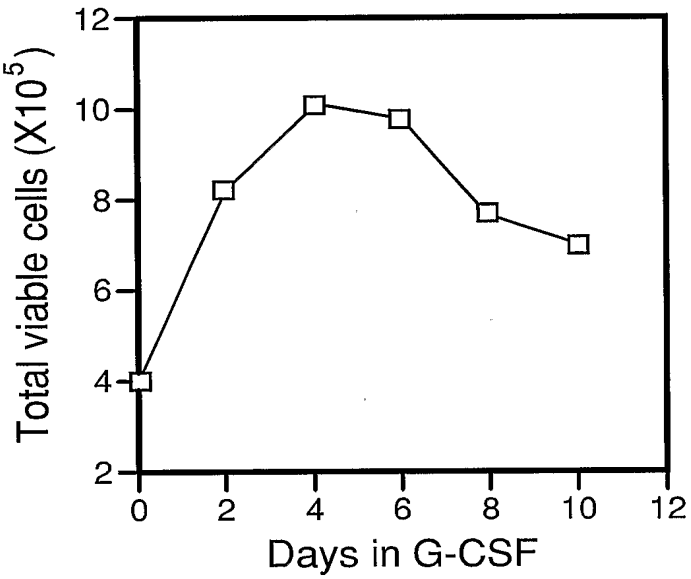


FIG. 4A

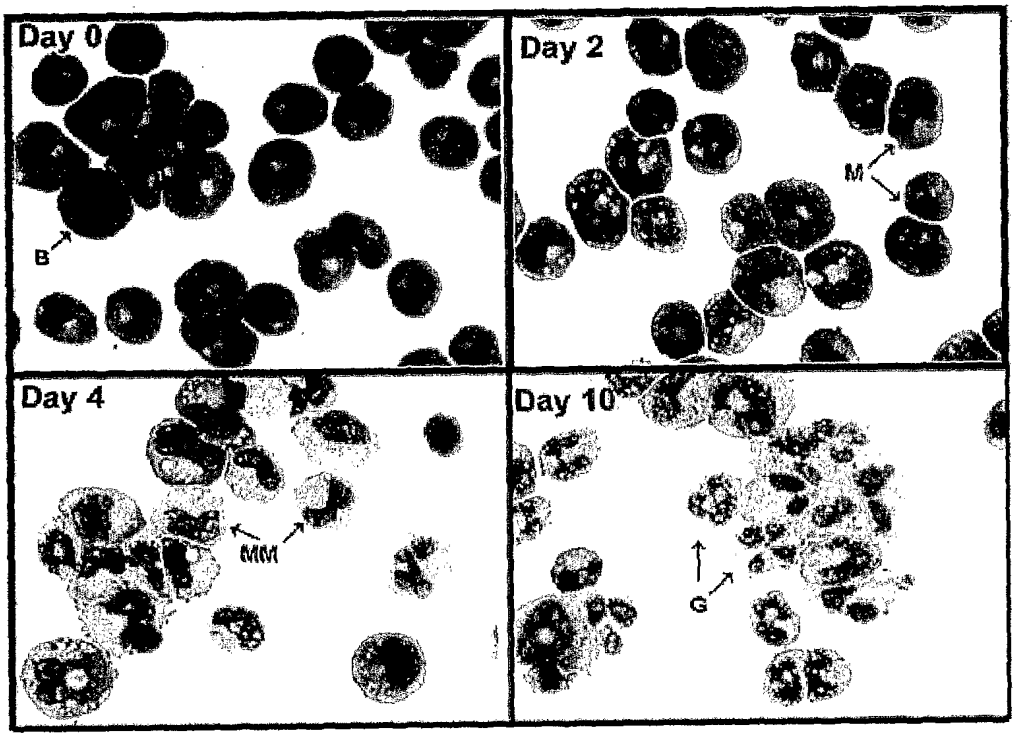


FIG. 4B

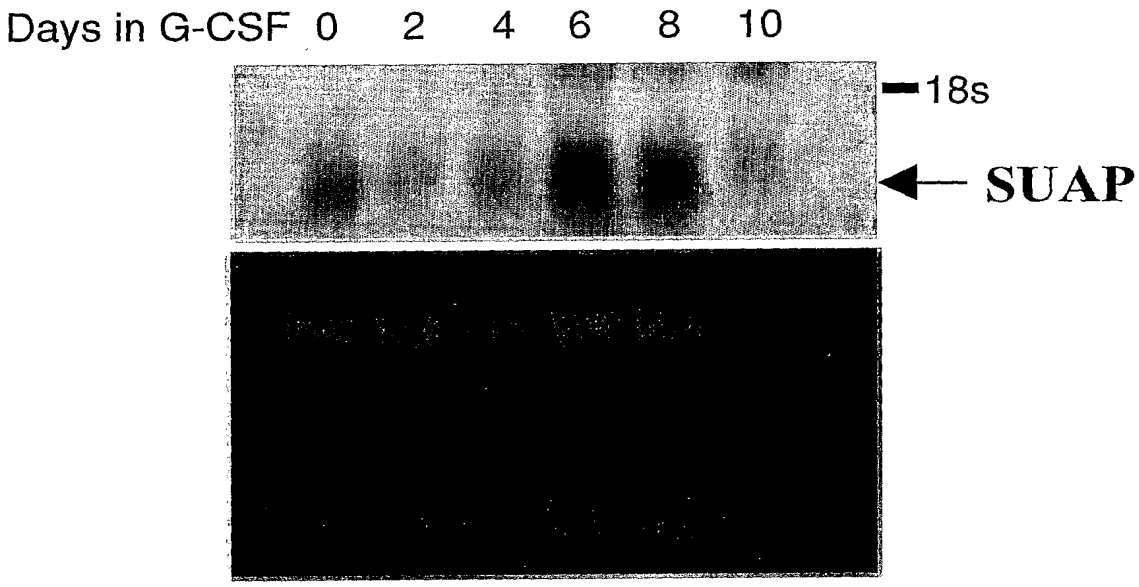


FIG. 5

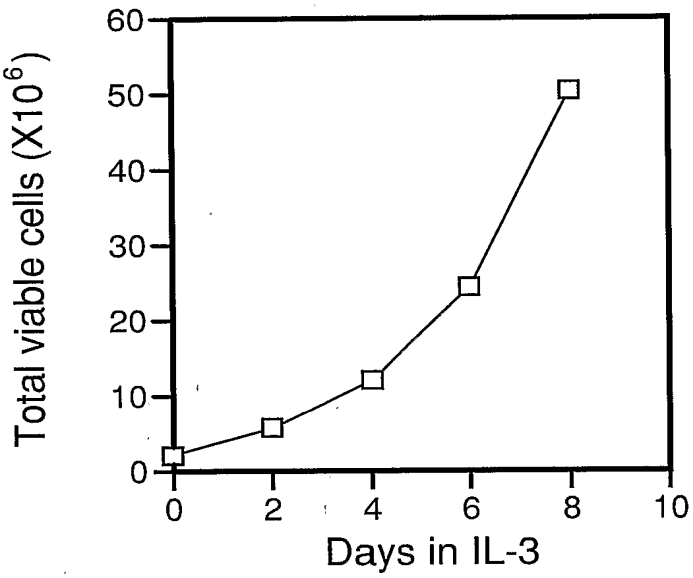

Total viable cells (X10^6)
60
50
40
30
20
10
0
0
2
4
6
8
10
Days in IL-3

FIG. 6A

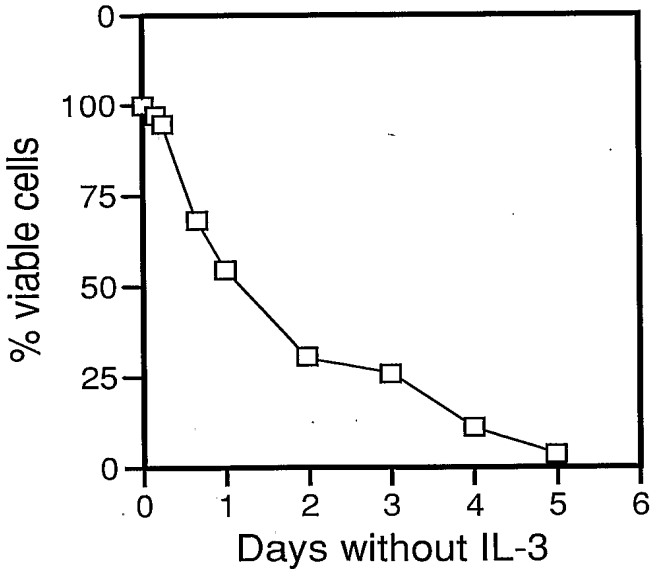

% viable cells
0
100
75
50
25
0
0
1
2
3
4
5
6
Days without IL-3

FIG. 6B

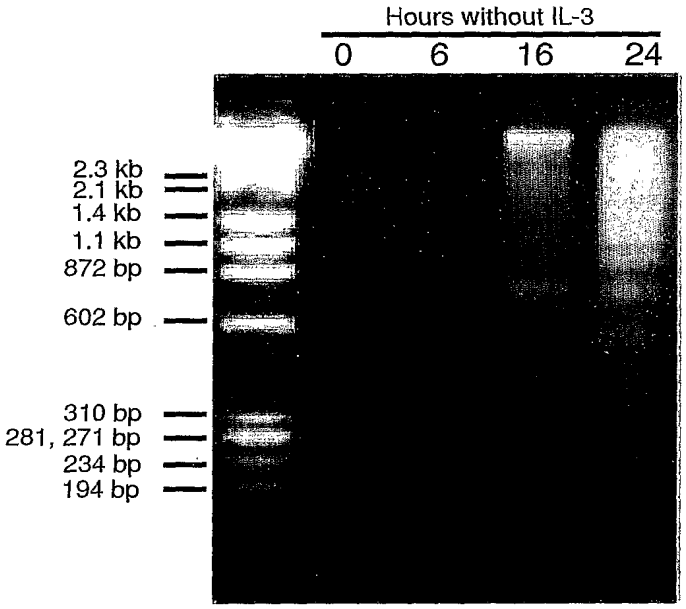

Hours without IL-3
0
6
16
24
2.3 kb
2.1 kb
1.4 kb
1.1 kb
872 bp
602 bp
310 bp
281, 271 bp
234 bp
194 bp

FIG. 6C

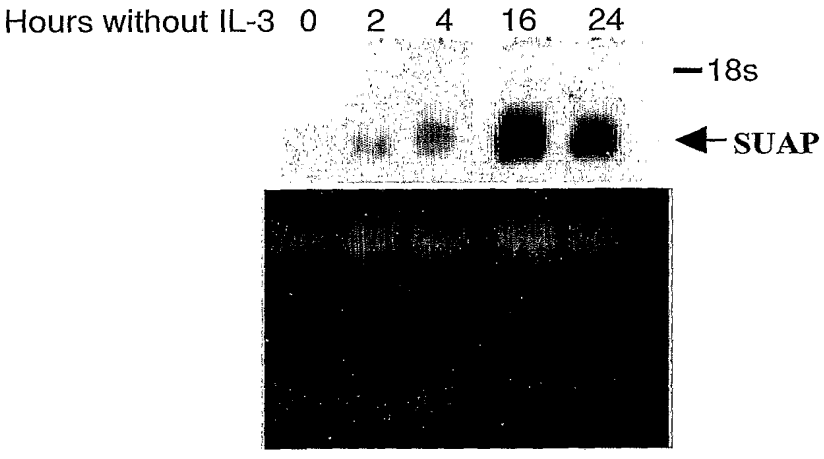

Hours without IL-3 0 2 4 16 24
18s
SUAP

FIG. 6D

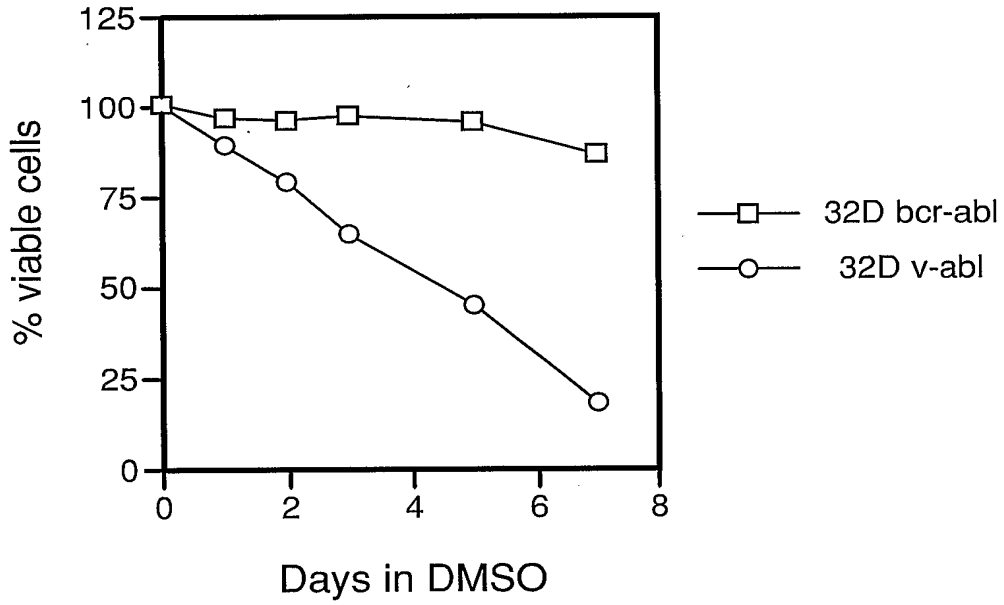

125
100
75
50
25
0
% viable cells
0
2
4
6
8
Days in DMSO
32D bcr-abl
32D v-abl

FIG. 7A

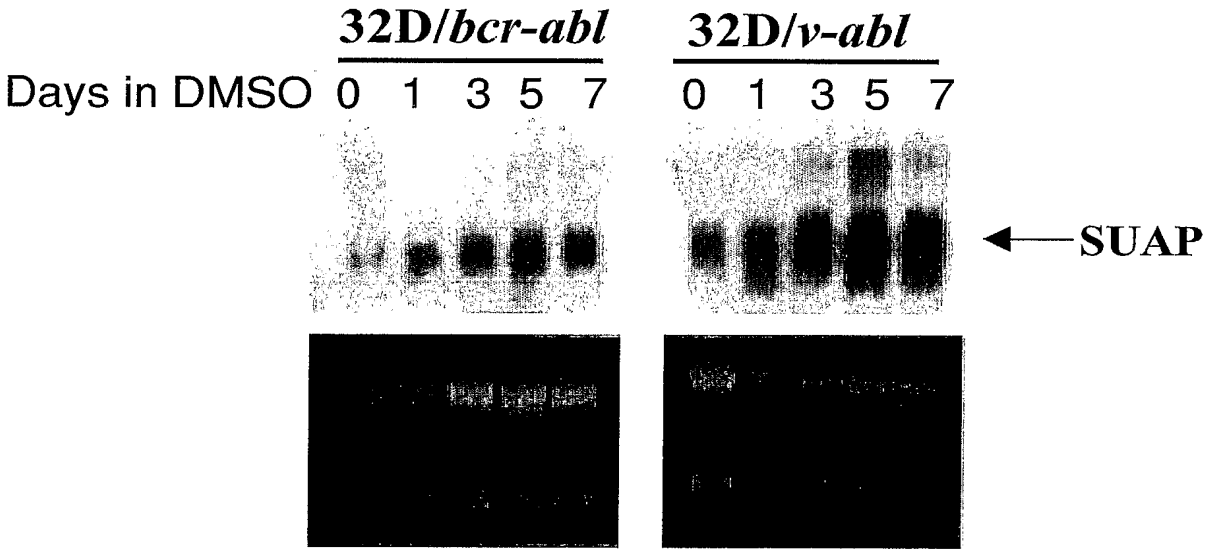


FIG. 7B

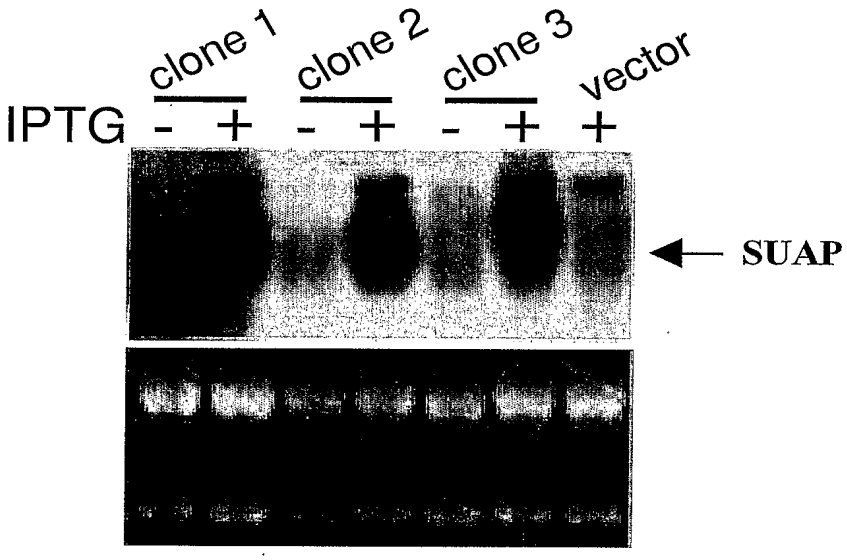


FIG. 8A

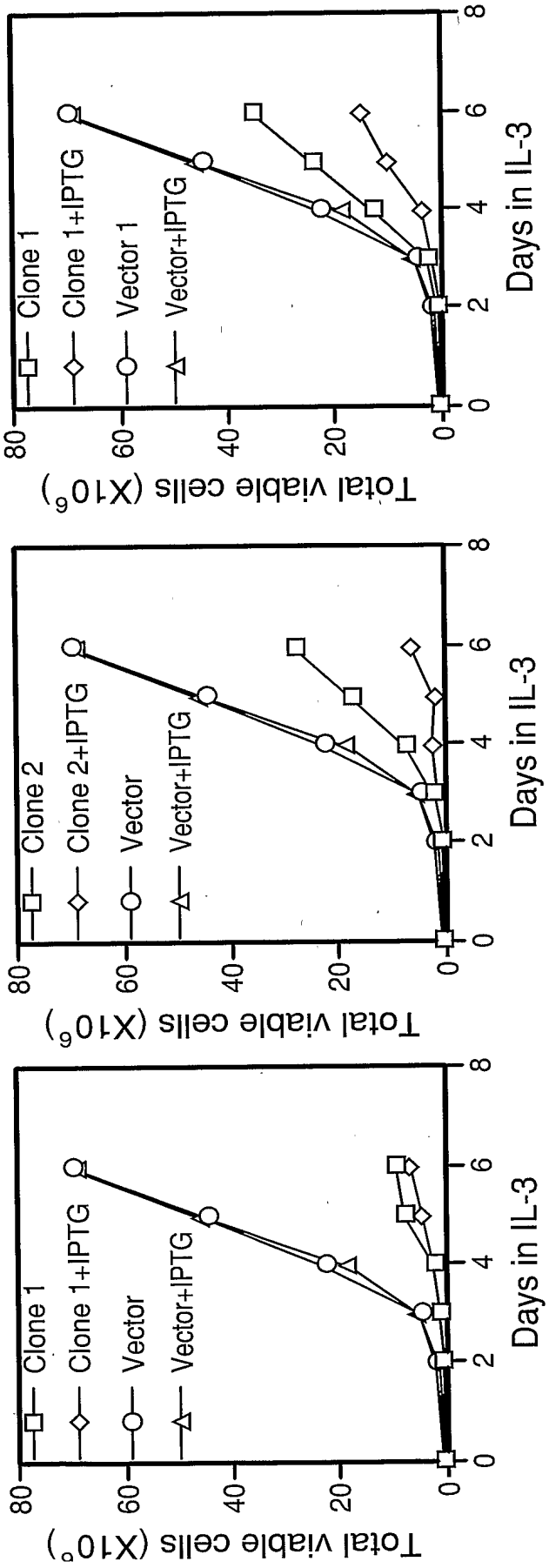

Total viable cells ($X10^6$)
0
20
40
60
80
Days in IL-3
0
2
4
6
8
Clone 1
Clone 1+IPTG
Vector
Vector+IPTG
Clone 2
Clone 2+IPTG
Vector
Vector+IPTG
Clone 1
Clone 1+IPTG
Vector 1
Vector+IPTG

FIG. 8B

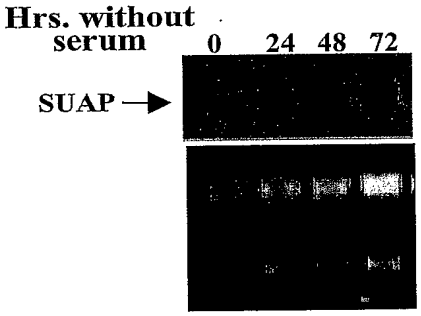


FIG. 9A

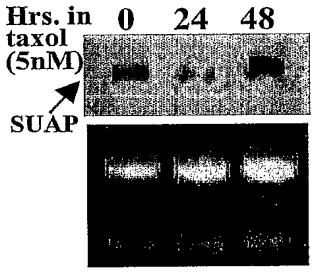


FIG. 9B

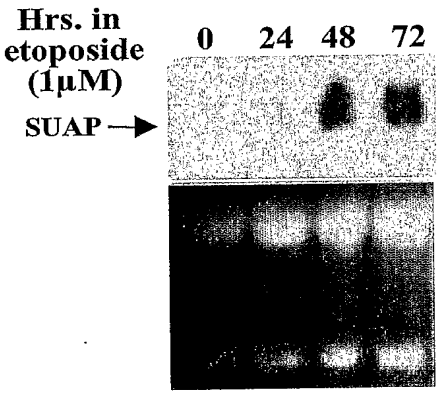


FIG. 9C

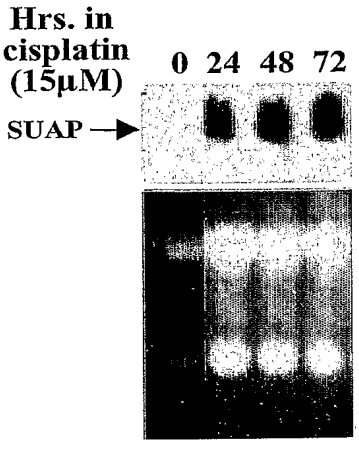


FIG. 9D

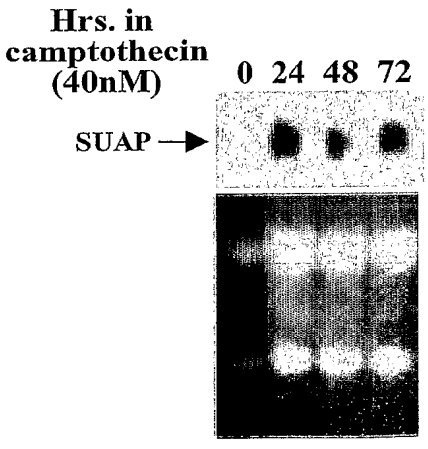


FIG. 9E

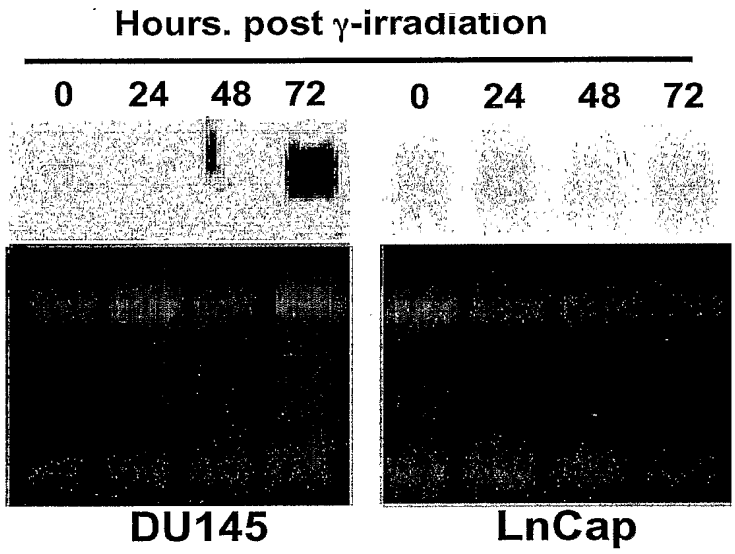


FIG. 10A

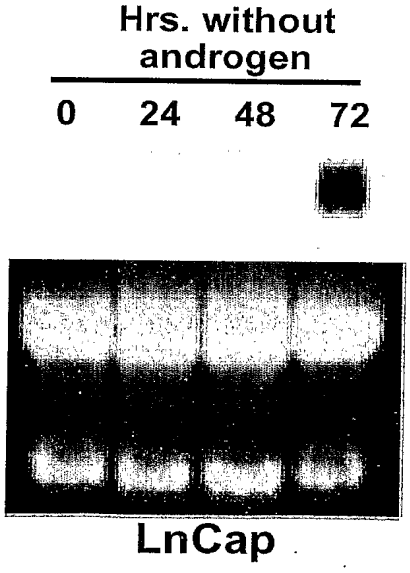


FIG. 10B

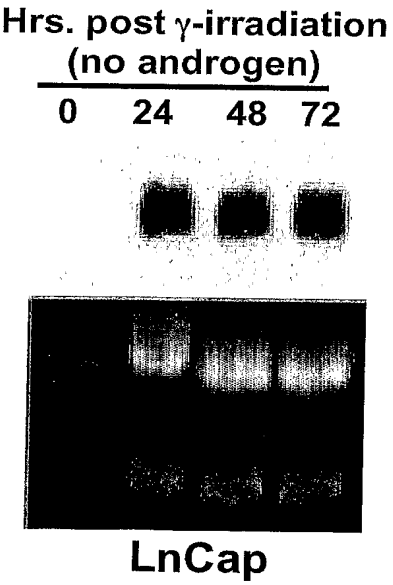


FIG. 10C

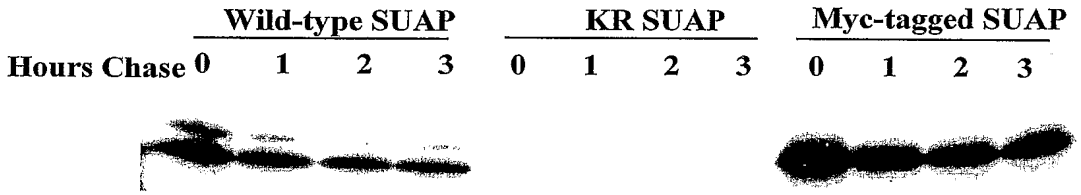

Wild-type SUAP
KR SUAP
Myc-tagged SUAP
Hours Chase 0 1 2 3 0 1 2 3 0 1 2 3

FIG. 11A

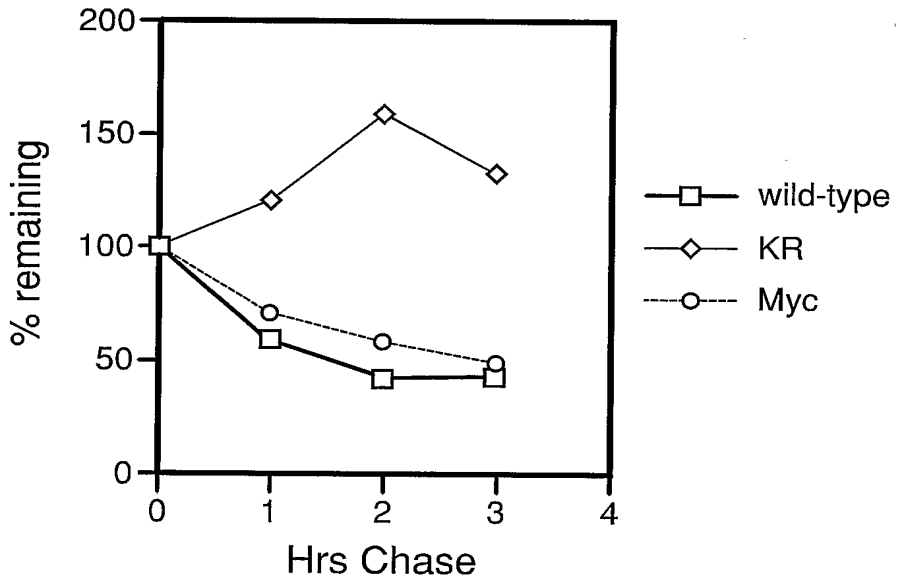


FIG. 11B

SEQUENCE LISTING

<110> Temple University - Of the Commonwealth System of Higher Education
Stacey J. Baker
E. Premkumar Reddy

<120> COMPOUNDS AND METHODS FOR INDUCING
GROWTH ARREST AND APOPTOSIS

<130> 6056-306 PC

<150> US 60/353,622
<151> 2002-01-31

<160> 16

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 1028
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<220>
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<222> (192)...(551)

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gcgaggcact gggctccctc tgctccccgt gggccgctcc ccgcgtgggg ccactgccct 180
tggcccccgc c atg gtg cgg att tca aag ccc aag acg ttt cag gcc tac 230
Met Val Arg Ile Ser Lys Pro Lys Thr Phe Gln Ala Tyr
1 5 10

ttg gat gac tgt cac cgg agg tat agc tgt gcc cac tgc cgt gct cac 278
Leu Asp Asp Cys His Arg Arg Tyr Ser Cys Ala His Cys Arg Ala His
15 20 25

ctg gcc aac cac gac gac ctc atc tcc aag tcc ttc cag ggc agt cag 326
Leu Ala Asn His Asp Asp Leu Ile Ser Lys Ser Phe Gln Gly Ser Gln
30 35 40 45

gga cgt gcc tac ctc ttc aac tct gta gtg aat gtg ggc tgc ggg cca 374
Gly Arg Ala Tyr Leu Phe Asn Ser Val Val Asn Val Gly Cys Gly Pro
50 55 60

gca gaa gag cgg gtg ctg ctg aca ggt ctt cat gct gtc gct gac atc 422
Ala Glu Glu Arg Val Leu Leu Thr Gly Leu His Ala Val Ala Asp Ile
65 70 75

cac tgt gag aac tgc aag acc act ttg ggc tgg aaa tat gag caa gcc 470
His Cys Glu Asn Cys Lys Thr Thr Leu Gly Trp Lys Tyr Glu Gln Ala

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        80                  85                  90

ttt gag agc agc cag aag tac aaa gag ggg aaa tac atc att gaa ctc   518
Phe Glu Ser Ser Gln Lys Tyr Lys Glu Gly Lys Tyr Ile Ile Glu Leu
     95                  100                 105

aac cac atg atc aaa gac aac ggc tgg gac tga cctagctgca tgtggcttca 571
Asn His Met Ile Lys Asp Asn Gly Trp Asp  *
110                 115

gcctggcctg gccgccaggg ggagccactg gtttcgctcc tcctgaaggg agctctggac 631
cctcagggcc cccacagggg aaggacccag ctcctgtaca tacattttat tgcatgcact 691
gtgaccttgg ggggagacaa agaggggggat tgtaccccca ccccctgcac ctccctgcga 751
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa agaattc                          1028

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<213> Mus musculus

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            20                  25                  30
His Asp Asp Leu Ile Ser Lys Ser Phe Gln Gly Ser Gln Gly Arg Ala
        35                  40                  45
Tyr Leu Phe Asn Ser Val Val Asn Val Gly Cys Gly Pro Ala Glu Glu
    50                  55                  60
Arg Val Leu Leu Thr Gly Leu His Ala Val Ala Asp Ile His Cys Glu
65                  70                  75                  80
Asn Cys Lys Thr Thr Leu Gly Trp Lys Tyr Glu Gln Ala Phe Glu Ser
                85                  90                  95
Ser Gln Lys Tyr Lys Glu Gly Lys Tyr Ile Ile Glu Leu Asn His Met
            100                 105                 110
Ile Lys Asp Asn Gly Trp Asp
        115


<210> 3
<211> 937
<212> DNA
<213> Homo sapiens

<220>
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<222> (143)...(502)
<223> human SUAP

<400> 3
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gccactgccc ccggcccccg cc atg gtg cgg att tca aag ccc aag acg ttt  172
                         Met Val Arg Ile Ser Lys Pro Lys Thr Phe
                          1               5                  10

cag gcc tac ttg gat gat tgt cac cgg agg tat agc tgt gcc cac tgc   220
Gln Ala Tyr Leu Asp Asp Cys His Arg Arg Tyr Ser Cys Ala His Cys
                 15                  20                  25

cgc gct cac ctg gcc aac cac gac gac ctc atc tcc aag tcc ttc cag   268
Arg Ala His Leu Ala Asn His Asp Asp Leu Ile Ser Lys Ser Phe Gln
             30                  35                  40

ggc agt cag ggg cgt gcc tac ctc ttc aac tca gtg gtg aac gtg ggc   316
Gly Ser Gln Gly Arg Ala Tyr Leu Phe Asn Ser Val Val Asn Val Gly
         45                  50                  55

tgc ggg cca gcc gag gag cgg gtg ctg ctg acc ggc ctc cat gct gtc   364
Cys Gly Pro Ala Glu Glu Arg Val Leu Leu Thr Gly Leu His Ala Val
     60                  65                  70

gcc gac atc cac tgc gag aac tgc aag acc act ttg ggc tgg aaa tat   412
Ala Asp Ile His Cys Glu Asn Cys Lys Thr Thr Leu Gly Trp Lys Tyr
 75                  80                  85                  90

gaa cag gcc ttt gag agc agc cag aag tac aaa gag ggg aag tac atc   460
Glu Gln Ala Phe Glu Ser Ser Gln Lys Tyr Lys Glu Gly Lys Tyr Ile
                 95                 100                 105

att gaa ctc aac cac atg atc aaa gac aac ggc tgg gac tga           502
Ile Glu Leu Asn His Met Ile Lys Asp Asn Gly Trp Asp  *
            110                 115

cccccgctcc cccgacgcat gtggctccag cccggcctgg ccgccaggga gcgccactgg 562
cttcccgcca cccgaaggga gctctggacc ctcagagccc ctgcagagga cggatctagc 622
tcctgtatat atattttatt gcatgcactg tgaccttggg gggagaacag aaggggacg  682
acgcccccgc acctcctgcg atctggctgg cttggatctc gtttttaacc cgttcctgcc 742
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aaaaaaaaaa aaaaa                                                  937
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<210> 4
<211> 119
<212> PRT
<213> Homo sapiens

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 1               5                  10                  15
Cys His Arg Arg Tyr Ser Cys Ala His Cys Arg Ala His Leu Ala Asn
            20                  25                  30
His Asp Asp Leu Ile Ser Lys Ser Phe Gln Gly Ser Gln Gly Arg Ala
        35                  40                  45
Tyr Leu Phe Asn Ser Val Val Asn Val Gly Cys Gly Pro Ala Glu Glu
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    50                  55                  60
Arg Val Leu Leu Thr Gly Leu His Ala Val Ala Asp Ile His Cys Glu
65                  70                  75                  80
Asn Cys Lys Thr Thr Leu Gly Trp Lys Tyr Glu Gln Ala Phe Glu Ser
                85                  90                  95
Ser Gln Lys Tyr Lys Glu Gly Lys Tyr Ile Ile Glu Leu Asn His Met
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Ile Lys Asp Asn Gly Trp Asp
        115
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<211> 1064
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<221> CDS
<222> (192)...(587)

<221> misc_feature
<222> (195)...(230)
<223> 36-nucleotide insert encoding 12-amino acid Myc epitope

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gcgaggcact gggctccctc tgctccccgt gggccgctcc ccgcgtgggg ccactgccct 180
tggcccccgc c atg gca tca atg cag aag ctg atc tca gag gag gac ctg  230
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gtg cgg att tca aag ccc aag acg ttt cag gcc tac ttg gat gac tgt   278
Val Arg Ile Ser Lys Pro Lys Thr Phe Gln Ala Tyr Leu Asp Asp Cys
     15                  20                  25

cac cgg agg tat agc tgt gcc cac tgc cgt gct cac ctg gcc aac cac   326
His Arg Arg Tyr Ser Cys Ala His Cys Arg Ala His Leu Ala Asn His
 30                  35                  40                  45

gac gac ctc atc tcc aag tcc ttc cag ggc agt cag gga cgt gcc tac   374
Asp Asp Leu Ile Ser Lys Ser Phe Gln Gly Ser Gln Gly Arg Ala Tyr
                 50                  55                  60

ctc ttc aac tct gta gtg aat gtg ggc tgc ggg cca gca gaa gag cgg   422
Leu Phe Asn Ser Val Val Asn Val Gly Cys Gly Pro Ala Glu Glu Arg
             65                  70                  75

gtg ctg ctg aca ggt ctt cat gct gtc gct gac atc cac tgt gag aac   470
Val Leu Leu Thr Gly Leu His Ala Val Ala Asp Ile His Cys Glu Asn
         80                  85                  90
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tgc aag acc act ttg ggc tgg aaa tat gag caa gcc ttt gag agc agc   518
Cys Lys Thr Thr Leu Gly Trp Lys Tyr Glu Gln Ala Phe Glu Ser Ser
     95                  100                 105

cag aag tac aaa gag ggg aaa tac atc att gaa ctc aac cac atg atc   566
Gln Lys Tyr Lys Glu Gly Lys Tyr Ile Ile Glu Leu Asn His Met Ile
110                 115                 120                 125

aaa gac aac ggc tgg gac tga cctagctgca tgtggcttca gcctggcctg      617
Lys Asp Asn Gly Trp Asp  *
                130

gccgccaggg ggagccactg gtttcgctcc tcctgaaggg agctctggac cctcagggcc 677
cccacagggg aaggacccag ctcctgtaca tacattttat tgcatgcact gtgaccttgg 737
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<213> Artificial Sequence

<220>
<223> Myc/SUAP fusion protein

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            20                  25                  30
Tyr Ser Cys Ala His Cys Arg Ala His Leu Ala Asn His Asp Asp Leu
        35                  40                  45
Ile Ser Lys Ser Phe Gln Gly Ser Gln Gly Arg Ala Tyr Leu Phe Asn
    50                  55                  60
Ser Val Val Asn Val Gly Cys Gly Pro Ala Glu Glu Arg Val Leu Leu
65                  70                  75                  80
Thr Gly Leu His Ala Val Ala Asp Ile His Cys Glu Asn Cys Lys Thr
                85                  90                  95
Thr Leu Gly Trp Lys Tyr Glu Gln Ala Phe Glu Ser Ser Gln Lys Tyr
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Lys Glu Gly Lys Tyr Ile Ile Glu Leu Asn His Met Ile Lys Asp Asn
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Gly Trp Asp
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<210> 7
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<220>
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<221> MUTAGEN
<222> 6, 8, 39, 83, 89, 99, 101, 104, 114
<223> substitution of Lys for Arg

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Cys His Arg Arg Tyr Ser Cys Ala His Cys Arg Ala His Leu Ala Asn
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His Asp Asp Leu Ile Ser Arg Ser Phe Gln Gly Ser Gln Gly Arg Ala
35 40 45
Tyr Leu Phe Asn Ser Val Val Asn Val Gly Cys Gly Pro Ala Glu Glu
50 55 60
Arg Val Leu Leu Thr Gly Leu His Ala Val Ala Asp Ile His Cys Glu
65 70 75 80
Asn Cys Arg Thr Thr Leu Gly Trp Arg Tyr Glu Gln Ala Phe Glu Ser
85 90 95
Ser Gln Arg Tyr Arg Glu Gly Arg Tyr Ile Ile Glu Leu Asn His Met
100 105 110
Ile Arg Asp Asn Gly Trp Asp
115

<210> 8
<211> 11
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<220>
<223> Protein Transduction Domain

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<210> 9
<211> 27
<212> DNA
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<220>
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<400> 9
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<210> 10
<211> 36
<212> DNA
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<220>
<223> nucleotide sequence encoding 12-amino acid Myc epitope

<400> 10
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<220>
<223> primer

<400> 11
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<220>
<223> primer

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<210> 13
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<220>
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aacggc 66

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<210> 16
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<213> Artificial Sequence

<220>
<223> primer

<400> 16
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(54) Title: COMPOUNDS AND METHODS FOR INDUCING GROWTH ARREST AND APOPTOSIS

(57) Abstract: Growth arrest and apoptosis in cells can be induced in cells which are resistant to apoptosis with SUAP and derivatives, homologs and analogs of SUAP. Detection of endogenous SUAP expression can also be used as a marker of apoptosis in cells undergoing apoptosis-inducing therapeutic treatments.

WO 2003/064616 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/02942

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/00; G01N 33/48, 33/53, 33/567, 33/574
US CL : 435/4, 7.1, 7.21, 7.23; 530/300, 350; 436/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/4, 7.1, 7.21, 7.23; 530/300, 350; 436/64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	BAKER, S.J. Small Unstable Apoptotic Protein, an Apoptosis-associated Protein, Suppresses Proliferation of Myeloid Cells. February 1, 2003, Vol. 63, pages 705-712.	1-50

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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